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ENDOCRINOLOGY

VOLUME 49 SEPTEMBER, 1951 NUMBER 3

ESTROGENIC, ANDROGENIC AND GONADOTROPHIC ACTIVITY IN WHEAT GERM OIL

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IN 1945, one of us (Levin), reviewed the need for differentiation between studies carried out with the tocopherols and wheat germ oil. The inability of workers employing tocopherols to repeat the early results obtained by those using wheat germ oil or wheat germ concentrates stimulated a search for other factors. Unpublished preliminary studies done in collaboration with the Department of Zoology, University of Illinois, indicated that wheat germ oil produced estrogenic and androgenic responses in rats. The present paper contains data demonstrating estrogenic, androgenic, and gonadotrophic activity in wheat germ oil.

Estrogenic substances have been reported from other plant sources—palm kernel, Butenandt (1933), pussy willow catkin, Skarzynski (1933), castor seed, Sahasrabudhe (1945), subterranean clover, Curnow (1948), licorice root, Costello (1950).

No mention of androgenic substances in plant material has been found in the literature. East, Underwood and Bennetts (1949) reported an increased weight of the accessory sex glands of male guinea pigs fed on a clover diet, but were able to obtain similar results by the injection of stilbestrol.

Friedman (1939, 1941), Fevold (1936) reported substances, protein in nature, revealing gonadotrophic activity.

It would not be expected to find gonadotrophic activity in a lipid substance. Yet histological findings in the ovaries of hypophysectomized rats studied in our work dealing with estrogenic activity in wheat germ oil, satisfied the criteria for gonadotrophic activity.

MATERIALS

The wheat germ oil was prepared by extraction of the wheat embryo with ethylene dichloride. The embryo was separated from hard wheat in

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the milling process and stored at 0° C. until processing. The free fatty acid of the finished oil was 8% or less (calculated as oleic). The processing was done in a manner to reduce oxidation to a minimum.

The rancid oil was prepared in a similar manner starting from wheat germ that had been rancidified by passing hot air (60° C.) through the germ for forty-eight hours. The oil showed no peroxide value by the Wheeler method (1932). The free fatty acid was 58%.

Distilled tocopherols and α-tocopherol acetate were used as a source of vitamin E.

A concentrate of the ketonic material in wheat germ oil was prepared by the Girard-Sandulesco (1936) reaction. This well known procedure was varied, in that isopropanol was used instead of methanol. The oily fraction thus obtained was diluted with sesame oil in a manner to obtain the desired dosage level based on the original wheat germ oil.

I. ESTROGENIC ACTIVITY

Three types of animals were used in the following experiments, (1) immature female rats, 21 days old, (2) adult spayed female rats, and, (3) immature hypophysectomized female rats, 21 days old. All animals were of the Sprague-Dawley strain and were maintained on a synthetic diet[1] for the duration of the experiment. It should be noted that the mortality was high in this study with the hypophysectomized rats. When the gonadotrophic study was initiated the usual diet of bread, milk and liver was given.

Experiment 1: Twenty-one day old female rats were divided into four groups. Group 1 received 0.5 cc. wheat germ oil three times each week; group 2 received 0.5 cc. rancid wheat germ oil three times each week; group 3 received 0.5 cc. of a 0.3% solution of distilled tocopherols in sesame oil three times each week; group 4 received 0.5 cc. sesame oil three times each week. All test substances were administered by subcutaneous injection. The experiment was considered terminated when the first animal in the control group exhibited vaginal introitus.

The results of this experiment are shown in Table 1.

Experiment 2: Two groups of rats were used to determine whether the hormonal activity of wheat germ oil is demonstrable when the oil is administered orally. One group of ten 21-day old animals received the synthetic diet containing 20% of wheat germ oil, and the second group of ten

[1] Casein, 25 lbs.; sucrose, 33 lbs.; dextrin, 33 lbs.; methyl linoleate, 0.5 lb., University of Illinois, Salt Mix 446—4 lbs., glycerol trilaurate, 4 lbs., B-vitamin mix, 90 gm.; (thiamine, 0.5 gm., riboflavin, 1.0, nicotinic acid, 2.0, pyridoxine, 0.5, PABA, 4.0, inositol, 20.0, vitamin K, 0.2, Ca-pantothenate, 2.0, dextrose, 369.8, total 400 gms.); choline, 45 gm.; vitamin A, 400,000 units; vitamin D, 80,000 units; vitamin E, 8.1 gm. (dl-tocopherol acetate); APF concentrate (12.4 mg. B_{12} per lb.) 35 gm.

TABLE 1. ESTROGENIC ACTIVITY OF WHEAT GERM OIL ON IMMATURE RATS (HARTMAN, 1945)

No. of animals	Treatment	Response*
Group 1 12	Wheat Germ Oil	8/12
Group 2 10	Rancid Wheat Germ Oil	0/10
Group 3 10	Distilled Tocopherols	0/10
Group 4 21	Control: Sesame Oil	1/21

* Expressed as the number of animals exhibiting vaginal introitus on the twenty-ninth day over the total number of animals in the group.

animals received the diet containing 20% of sesame oil. On the day the first animal of the control group exhibited vaginal introitus five of the experimental animals exhibited introitus and vaginal smears showed them to be in estrus.

Experiment 3: Two groups of 21-day old hypophysectomized female rats were used. One group received 2.0 cc. of wheat germ oil; one group received 2.0 cc. of sesame oil. The subcutaneous injections of the test substances were begun on the tenth post-operative day and were continued every second day until a total of four 0.5 cc. injections had been administered. The animals were sacrificed on the ninth day and the uteri removed and weighed on a Roller-Smith torsion balance. The results of this experiment are summarized in Table 2.

TABLE 2. EFFECT OF WHEAT GERM OIL ON UTERI OF HYPOPHYSECTOMIZED RATS

No. of animals	Treatment	Uterine wt. mg./100 g.
20	Wheat Germ Oil	63.5 ± 2.3
20	Sesame Oil	29.7 ± 0.92

Experiment 4: To determine quantitatively the estrogenic activity in wheat germ oil four groups of 12 spayed mature rats were used. The design of the assay followed that of Curtis *et al.* (1947). U.S.P. reference standard estrone was used. The dosages for the high and low standard were 22.4 I.U. and 14.1 I.U. respectively. The unknown was the Girard fraction described above (Materials). It was diluted to maintain the same dilution factor on both the standard and the unknown. The two dose method of assay was used and the first smears were taken at 48 hours. At 72 hours all the animals in both the unknown groups were in estrus.

The experiment was set up again using a lower wheat germ oil equivalent on the unknown and the results are given in Table 3. Twenty animals

were employed on each point. The high unknown group received the equivalent of 2.5 cc. of wheat germ oil. The calculated potency of the wheat germ oil is thus shown to be 9 I.U. of estrone per cc.

TABLE 3. ESTROGENIC ASSAY OF WHEAT GERM OIL

Group	Dosage in I.U.	Log dose	Response	Degrees θ
S_H	22.4	1.350	18/20	71.6
S_L	14.1	1.149	12/20	50.8
U_H	22.4	1.350	17/20	67.2
U_L	14.1	1.149	15/20	60.0

Dilution factor = 1
$V = 67.2 + 60.0 - 71.6 - 50.8 = 4.8$
$W = 67.2 + 71.6 - 60.0 - 50.8 = 28.2$
Potency = 104.0
$Z = V^2 + W^2 = 23.04 + 812.0 = 835.04$
Ratio = .11
Standard error = $104.0 \times .11 = 11.4$
Potency = $104.0 \pm 11.4\%$ of standard

II. ANDROGENIC ACTIVITY

Two methods were used in the measurement of androgenic effect, (1) the chick comb growth method, as reported by Frank (1942), and by Dorfman (1948), and (2) the seminal vesicle response method of Moore (1929).

The Frank method gives a numerical result of the amount of androgenic activity calculated as androsterone in an oil; the Dorfman method depends on comparison of the comb-body weight ratio with a standardized curve. Dorfman obtains linearity of response over a fairly wide range including those concentrations found in wheat germ oil.

The increase in seminal vesicle weight of castrate animals under the influence of androgens is well known. For example, Mathieson and Hays (1945) base a quantitative assay on the probit (two level dose) application of this method. Their assay involves a higher level of androgen than that present in wheat germ oil. The method of Moore (1929) was found suitable for our purpose. In order to put this method on a quantitative basis the statistical design of Bliss (1935) was used.

In addition to the two weight-response methods mentioned, a histological study was made of the pituitary glands of the castrate rats.

In all comb growth methods day-old White Leghorn chicks were used. The Frank method, used in earlier work, requires the chicks to be weighed at the beginning and end of the experimental period. The birds are sexed after they are sacrificed; the response in terms of micrograms of androsterone is calculated for a group by a formula involving comb weight, initial and terminal body weights, and sex. It is not possible to calculate

standard errors for a group using this method. The results of eight separate assays run by this method are shown in Table 4.

TABLE 4. EFFECT OF WHEAT GERM OIL ON CHICK-COMB GROWTH AS MEASURED BY THE FRANK METHOD

Sesame oil		Testosterone propionate 10μg./cc.		Wheat germ oil	
No. of birds	μg. of androsterone per cc.	No. of birds	μg. of androsterone per cc.	No. of birds	μg. of androsterone per cc.
21	0	21	36.0	21	27.0
21	4.5	20	38.6	21	25.2
21	7.8	21	35.8	21	29.0
22	0	21	32.2	21	15.5
22	0	22	40.0	19	31.0
22	0	21	34.0	21	14.0
20	3.0	21	37.0	20	20.0
20	5.4	17	26.0	17	30.0
				20	26.0
				18	24.0
				19	17.0

It will be noted that the testosterone at the 10 microgram per cc. level gives an average response equivalent to 35 micrograms of androsterone per cc., a ratio of 3.5 to 1 instead of the usually accepted ration of 7:1 (Dorfman, 1948). These birds were maintained on a purified diet, which may have affected the result. The figures, therefore, are considered as being qualitative.

A number of assays have been carried out with wheat germ oil, using the method of Dorfman of applying the oil topically to the combs of commercially sexed White Leghorn chicks (cockerels) fed commercial chick diet. Despite a single source of supply of birds, and every effort to maintain a uniform environment, a large variation occurs in the response to testosterone propionate applied by inunction. Each figure in Table 5 represents the average response of 20 to 22 birds. There appeared to be no noticeable seasonal trend.

TABLE 5. EFFECT OF WHEAT GERM OIL ON CHICK COMB GROWTH AS MEASURED BY THE DORFMAN METHOD

Treatment	Response, mg. comb wt./100 g. body wt.
Sesame Oil	$39 \pm 2.6, 39 \pm 2.5, 37 \pm 2.5, 36 \pm 2.9, 37 \pm 2.7$ $37 \pm 2.2, 37 \pm 2.4$
Wheat Germ Oil	$47 \pm 2.2, 50 \pm 3.6$
Test. prop. 2.5 μg./cc.	45 ± 1.9
Test. prop. 5 μg./cc.	54 ± 2.9
Test. prop. 10 μg./cc.	62 ± 4.1
Test. prop. 15 μg./cc.	$49 \pm 3.0, 70 \pm 5.3, 57 \pm 4.0, 52 \pm 2.2, 62 \pm 4.8$

The response of the seminal vesicles in the orchidectomized rat was also used as a criterion of androgenic activity. The statistical design of Bliss (1935) for a two-dose assay was used in setting up the experiment.

Four groups of Sprague-Dawley rats were orchidectomized at 28 days of age. Two groups, S_H and S_L, received 0.5 cc. of testosterone propionate, 5μg/cc. and 2.5μg/cc. respectively, every second day until a total of 8 injections had been given. Two groups, U_H and U_L, received 0.5 cc. wheat germ oil undiluted and diluted 1:1 with sesame oil respectively, every second day until a total of 8 injections had been given. Two days following the final injections the animals were sacrificed, the seminal vesicles removed and weighed on a Roller-Smith torsion balance. The pituitaries were removed and placed in FAA solution for histological study.

The seminal vesicle weights are given in Table 6.

TABLE 6. EFFECT OF WHEAT GERM OIL ON SEMINAL VESICLES OF CASTRATE RATS

Group	No. of animals	Treatment	Seminal vesicles in mg.
S_H	9	Test. Prop. 5 μg./cc.	23.7 (18.6–32.4)
S_L	9	Test. Prop. 2.5 μg./cc.	12.9 (9.9=18.2)
U_H	9	Wheat Germ Oil	38.7 (21.3–59.4)
U_L	9	Wheat Germ Oil (1:1)	20.4 (10.3–27.4)
Control	6	Sesame Oil	6.1 (5.4–10.8)

The character of the response between the unknown and known groups precludes considering this experiment as a quantitative assay. Since the precise nature of the active material in wheat germ oil is not known, and the Bliss design applies only when the same material is given to standard and unknown groups, this result is not surprising. Yet the results indicate that wheat germ oil contains a minimum of androgenic activity equivalent to group S_H, namely 5μg. of testosterone propionate per cc. of wheat germ oil.

Histological examination of the pituitaries of these animals showed a marked diminution in the number of "castration cells" in the anterior lobe over those seen in the controls.

In our studies of estrogenic activity it was shown that the Girard procedure concentrated active estrogenic fractions of wheat germ oil. Isopropanol was used as the solvent. It was found that this form of Girard

concentrate removed a small part of the androgenic activity from wheat germ oil. However, a Girard fractionation made by using absolute methanol provided a ketonic fraction which yielded more of the androgenic activity. One hundred grams of wheat germ oil yielded approximately 0.5 gram of a crude ketonic fraction. This was dissolved in 10 cc. of sesame oil and applied topically to chick combs in the same manner that the original wheat germ oil was used. Two separate preparations gave responses of 54 ± 3.1 mg. and 48 ± 4.4 mg. comb weight per 100 grams of body weight, in comparison with 36 ± 2.9 mg. and 37 ± 2.3 mg. for sesame oil controls respectively. Statistical analyses show that the difference between the wheat germ oil and control groups, as well as that between the Girard concentrate and control groups is highly significant. While the response obtained does not indicate quantitative recovery of the active material from the original oil, it does indicate the ketonic nature of the active substance.

III. GONADOTROPHIC ACTIVITY

In Experiment 3 (Table 2) the weights of the uteri of hypophysectomized rats were used to evaluate estrogenic activity in wheat germ oil. The ovaries and uteri of the rats used in this experiment were examined histologically.

It was observed that there was a marked stimulation of follicular maturation and repair of the "deficiency cells" in the interstitial tissue. Corpus luteum formation was also observed in several of the animals. From the absence of any progestational proliferation in the uterine endometrium it must be assumed that these corpora lutea were non-functional.

Similar effects were observed in ovaries of hypophysectomized rats given wheat germ oil orally, to the extent of ten per cent of the diet.

A small group of hypophysectomized rats fed rancid wheat germ oil revealed ovarian and uterine histology similar to the controls. Negative results were apparent in the ovaries and uteri of hypophysectomized rats treated with tocopherols (Table 7).

TABLE 7. ORAL ADMINISTRATION OF WHEAT GERM OIL TO HYPOPHYSECTOMIZED RATS

No. of animals	Treatment	Average uterine weight mg./100 g.
4	Sesame Oil	24.5
10	Wheat Germ Oil	80.8
7	Rancid Wheat Germ Oil	22.7
8	0.6% d,l, α-tocopherol Acetate	27.9

The following experiment repeated the study of the effect of wheat germ oil on the ovaries and uteri of hypophysectomized rats using the Girard ketonic fraction of wheat germ oil.

Experiment 1: Three groups of 21-day old hypophysectomized female rats were used. The Girard ketonic fraction was diluted with sesame oil to provide two levels; one, equivalent to 1.5 cc. of wheat germ oil per cc., the other, equivalent to 3.0 cc. of wheat germ oil per cc.

The animals received 0.1 cc. of these preparations beginning on the 16th post-operative day and continuing every second day until a total equivalent of 0.75 cc. of wheat germ oil was administered on the low level; and 1.5 cc. on the high level.

On the thirteenth day after starting the injections, the animals were sacrificed, the uteri and ovaries were removed, weighed on a Roller-Smith torsion balance and fixed in FAA solution. The organ weights are given in Table 8.

TABLE 8. EFFECT OF WHEAT GERM OIL ON HYPOPHYSECTOMIZED RATS

No. of animals	Treatment	Uterine weight mg./100 g.	Ovarian weight mg./100 g.
25	Sesame Oil	31.0±0.81	9.2±0.44
22	Girard fraction 1 cc. ≏1.5 cc. Wheat Germ Oil	218 ±2.27	16.2±0.58
21	Girard fraction 1 cc. ≏3.0 cc. Wheat Germ Oil	235 ±2.95	11.0±1.06

The increased ovarian weights as shown in hypophysectomized rats treated with the low level of the Girard fraction (Table 8) is an indication of gonadotrophic activity. The confirmation of this phenomenon is shown in the histological examination of the ovaries and uteri.

OVARIAN HISTOLOGY

No large antrum-containing follicles were observed in the controls. The follicles remaining were largely atretic. Primordial follicles were in poor state of preservation. Typical agmination of the nuclear chromatin of the interstitial tissue was present. The average maximum follicle size was 200μ. See Figure 1.

In the ovaries of the animals receiving the low level of Girard concentrate there was a marked increase in the number of follicles having a diameter greater than 300μ. Some of these follicles showed beginning antrum formation. The majority of the follicles were characterized by a marked increase in the membrana granulosa, as supported by the increase in the number of mitotic figures seen in the granulosa cells. There was a marked repair of the "deficiency cells" in many of the ovaries but this response was noticeably lacking in uniformity. The primordial follicles were in good

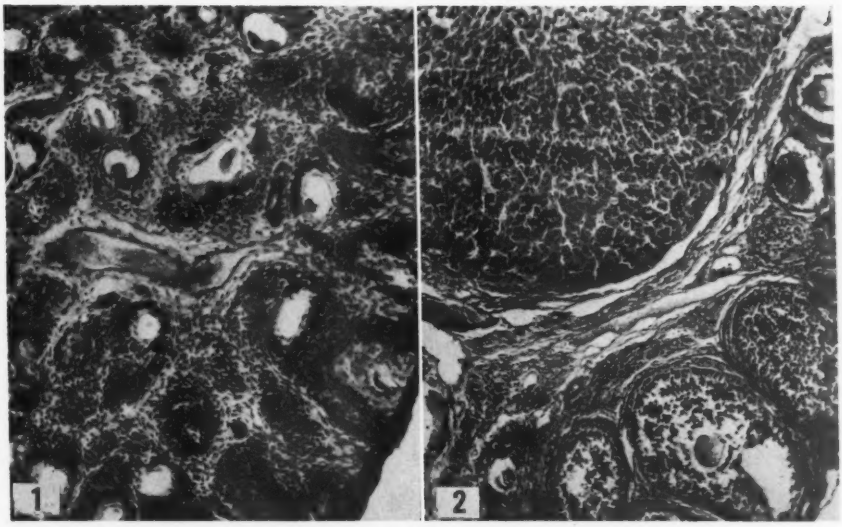

FIG. 1. Ovary of hypophysectomized rat (control). Hematoxylin-eosin. ×100.
FIG. 2. Ovary of hypophysectomized rat treated with wheat germ oil concentrate. Note corpus luteum and repair of "deficiency" cells. Hematoxylin-eosin. ×100.

state of preservation. In a moderate number, mitotic figures could be seen in the germinal epithelium. See Figure 2.

In the animals receiving the high level concentrate, the average maximum follicle size was not as great as in the low level (250–275μ) and a moderate number of follicles exhibited marked atresia. There was little or no repair of the "deficiency cells" of the interstitial tissue.

UTERINE HISTOLOGY

On the low level all uteri exhibited pronounced hyperplasia of the myometrium and increased vascularity of the stroma. In about 60% of the animals moderate progestational proliferation was observed. See Figure 6.

On the high level the uteri showed a typical enlargement to be expected from estrogen stimulation. See Figures 4 and 5. In only two of the animals was there any progestational proliferation.

Experiment 2: To confirm the gonadotrophic-like effect observed in the hypophysectomized rats the Friedman test was applied, using the Girard fraction. Sixteen Friedman-test does were procured from a local hospital supply house. Twelve received intravenous injections of 5 cc. of a suspension composed of 12 cc. of crude Girard fraction, 12 cc. of propylene glycol, and 88.5 cc. of 0.9% saline. This was equivalent to 8 cc. of original wheat

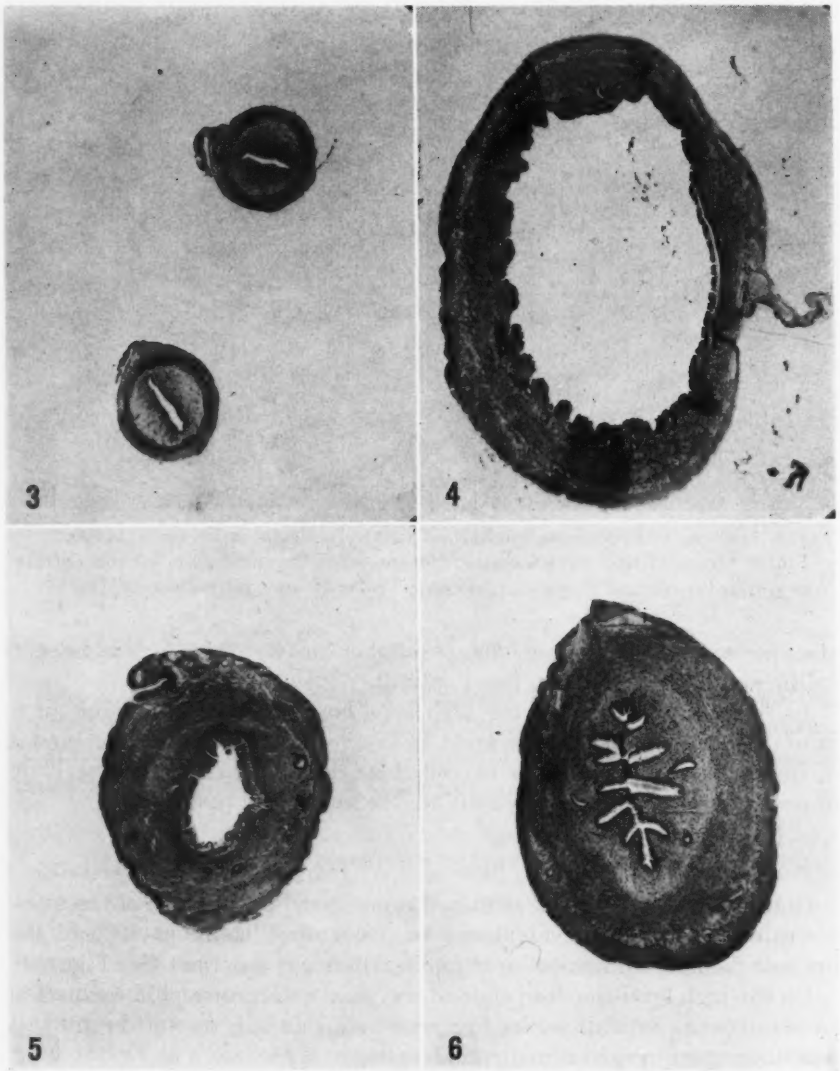

FIG. 3. Uterus of hypophysectomized rat (control). Hematoxylin-eosin ×50.

FIGS. 4 and 5. Uterus of hypophysectomized rat treated with wheat germ oil concentrate. Hematoxylin-eosin ×50.

FIG. 6. Uterus of hypophysectomized rat treated with wheat germ oil concentrate. Note the progestational prolifieration. Hematoxylin-eosin ×50.

germ oil. The rabbits were sacrificed in groups at 48, 60 and 72 hours following injection.

Of 8 animals sacrificed after 48 hours, 7 had corpora hemorrhagica in the ovaries. Two animals at 60 hours and 2 at 72 hours likewise showed a positive response. Four animals received a similar suspension using sesame oil instead of the wheat germ oil concentrate. The results were negative.

In a second experiment of this series, two 7-day post-partum rabbits were used. The same amount of the suspension of wheat germ oil concentrate used in the experiment with immature rabbits showed a positive response 60 hours after injection in the post-partum rabbits.

DISCUSSION

It has been found that wheat germ oil, solvent extracted from fresh wheat germ with ethylene dichloride elicits estrogenic response. This was shown by (a) eliciting vaginal introitus in immature female rats, with wheat germ oil given by injection and orally; (b) increased uterine weight in hypophysectomized rats; (c) development of estrus in ovariectomized rats. The active principle is ketonic.

The biological evidence for estrogenic substances from plant sources previously reported in the literature has been obtained by subcutaneous injection except for that found in subterranean clover, (Curnow, 1948). Here again it is shown that a plant estrogenic substance is effective when administered orally. The use of a "synthetic" diet was indicated because of the possible destruction of fat soluble nutritional factors in the presence of rancid food in the diet (Kennelly and Quackenbush, 1946). The likelihood of destruction is supported by our finding that hormonal activity is absent in oil from rancid wheat germ. It is evident that oxidative deterioration need not be extensive inasmuch as peroxides were not detectable in the rancid oil.

Androgenic activity has been demonstrated in (1) a plant substance and in (2) wheat germ oil. We have been unable to find a report of androgenic activity of vegetable origin.

Androgenic activity was shown by (a) the chick comb growth method, (b) the seminal vesicle response method, (c) the diminution of "castration cells" in the anterior lobe of the pituitary of the castrate rat.

The active principle is ketonic.

We have demonstrated for the first time, (1) gonadotrophic activity in a lipid substance; (2) gonadotrophic activity in wheat germ oil. The active principle is ketonic. Gonadotrophic activity was demonstrated by (a) the repair of the deficiency cells in the ovaries of hypophysectomized rats, (b) the formation of corpora hemorrhagica in the rabbit, and (c) the increased

weight of ovaries of animals on the lower level of the Girard concentrate (Table 8).

The diminished response on the higher level of the wheat germ oil concentrate might be explained by the fact that a similar phenomenon occurs when pituitary gonadotrophins are evaluated. The presence of an antigonadotrophic substance in wheat germ oil should not be excluded. The source of follicular stimulation will remain in doubt until the gonadotrophic substance is separated from the estrogenic substance.

In this respect, Pencharz (1940) and Williams (1944, 1945) have shown that estrogen administered to hypophysectomized rats will maintain the weights of the ovaries. However, it appears quite unlikely that the ovarian response observed in our animals would be due primarily to estrogen stimulation because (1) the total amount of estrogen administered was significantly below the levels employed by Williams, and Pencharz, and (2) injections of our animals were not initiated until the sixteenth post-operative day, when the ovaries would reveal an advanced stage of atrophy.

The progestational proliferation observed in the uteri of the rats must be considered gonadotrophic in origin.

Extensive experiments using both Corner-Allen and McPhail assays were carried out to determine whether wheat germ oil contains progestational activity. Such activity was clearly demonstrated in many instances. However, in approximately an equal number of animals the results were negative. Thus, it cannot be said that a progestational substance is present as such. We have no explanation for these equivocal results.

SUMMARY

Estrogenic, androgenic, and gonadotrophic activity have been demonstrated in wheat germ oil, solvent extracted at low temperatures with ethylene dichloride.

These effects were not found in rancid wheat germ oil. Tocopherols were likewise ineffective.

The active principles are ketonic.

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INCOMPLETE HEPATIC INACTIVATION OF HORMONE PRODUCED BY THE INTRASPLENICALLY GRAFTED OVARY IN THE MOUSE[1,2,3]

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INTRODUCTION

UNTIL very recently the opinion has prevailed that the animal bearing an ovarian graft in the spleen is, in terms of the hormone content of its systemic circulation, a physiological castrate. Considerable amounts of either estrogen or progesterone are removed from the blood stream when the hormone is placed in the portal drainage area (Segaloff and Dunning, 1946, Engle, 1946).

Data of a quantitative nature concerning the removal of sex hormone by the liver have been obtained in experiments with the castrated graft-bearing chick (Bernstorf, 1948). While the present investigation was in progress, data obtained with the rat have been published by both Werthessen and Field (1950), and by Greep and Jones (1950) showing agreement with data presented here. The present work gives indication both by organ weight comparison and by microscopic methods that a significant amount of active hormone passes into the systemic circulation from an intrasplenically grafted ovary in the mouse.

METHOD

Two series of three subgroups each of albino Swiss female mice were used in this work. The number of animals in each experimental group is shown in Table 1. The normal groups were given no special treatment; the castrates were merely spayed and the graft animals were bilaterally spayed and given an autoplastic intrasplenic graft. Mice of series A were operated at approximately 70 days of age and sacrificed at approximately 343 days. Series B graft-bearing animals were 52–62 days of age at operation while the castrates, although of the same group, were spayed later at 170–180 days of age. All series B animals were sacrificed at 199–209 days.

All animals within a series were operated within a 1 to 3 day period. As a rule, bleeding did not occur from the spleen and nearly every graft "took." The number of animals

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with adhesions between the spleen and the body wall was less than 2 to 3 per cent of the total number receiving grafts.

Food was removed a few hours preceding sacrifice. The mice were weighed and then anesthetized with ether. The uterus and vagina were removed together and weighed as a unit. The graft was cleaned of lienal tissue and any cystic follicles were burst before the graft was weighed. The pituitaries were weighed individually in a tightly stoppered tared vial. Any animal having an adhesion between the spleen and body wall was discarded completely. Organ weights were computed in terms of body weight for each animal and the "t" values were found for the averages of these values by the method of Snedecor (1946). A portion through the middle of the vagina and through the caudal portion of one uterine cornu from mice of series B was fixed in Bouin's, sectioned at 6 microns and stained with hematoxylin and either Mallory's triple connective tissue or Masson trichrome stains. The graft or an ovary from the first few animals killed in both the entire and graft-bearing groups was stained in a similar manner after sectioning at 8 microns.

Microscopic observation was made of the general condition of the grafts and the amount of luteal and follicular material present. The vaginae were checked for mucification and amount of cornification. Also, the appearance of the uterine stromal nuclei was observed using the method of Hooker and Forbes (1947) for progesterone assay.

DATA AND DISCUSSION

From Table 1, it is immediately apparent that the uterine (plus vagina) weight of either the graft-bearing or the castrate animal is considerably

TABLE 1. SUMMARY OF SACRIFICE DATA FOR NORMAL, GRAFT-BEARING AND CASTRATE MICE

Series	Group	Number in group	Average body wt. in grams	Average organ weights (mg./g. body wt.)		
				Uterus-vagina	Ovaries or graft	Pituitary
A	Normal	18	27.5	4.807	—*	—*
	Castrate	15	33.1	1.354	—	—
	Graft-bearing	15	31.5	2.554	7.154	—
B	Normal	16	31.6	6.025	0.494	0.0735
	Castrate	16	31.7	1.472	—	0.0715
	Graft-bearing	33	33.0	3.012	1.691	0.0679

* Tissues not weighed.

less than that of the normal mouse. However, the weights of the first two groups are not equally reduced. The uterine weights of the graft-bearing animals exceed those of the castrates and these differences were found to be statistically significant at the one per cent level. Thus the uterine weight of the graft-bearing, while significantly (at the 1% level) less than that of the normal control animal, is significantly greater than that of the spayed control. This is the same type of relationship that was described earlier in the male chick (Bernstorf, 1948) and in the female rat (Werthessen and Field, 1950; Greep and Jones, 1950) and indicates that an amount of ovar-

ian hormone sufficient to bring about this increase in uterine weight escaped inactivation in the liver.

Because of the macroscopic and microscopic appearances of many grafts and because of unpublished data obtained from the biological assay of urinary estrogen in female graft-bearing mice, the author believes that during at least part of the functional life of the intrasplenically grafted ovary, its secretory output is as great as that of the normal pair of ovaries. If this is correct, these data are evidence that the liver has removed or inactivated a significant amount of hormone.

The similarity between the uterine weight differences seen in animals of the two series is noteworthy because the mice were purchased at different times and were of different ages. Each percentage difference for series B groups is consistently about 4 per cent greater than the corresponding value for the series A groups. The fact that the values for the younger animals (series B) are slightly higher than those of the older animals may indicate an effect of age difference upon the secretory activity of the graft. Apparently the small differences in the pituitary weights of the three groups are of no significance.

The graft-bearing animal with only one of its two gonads present is in a sense a unilateral castrate, but actually there seems to be a great difference. Although the remaining testis in the unilaterally castrated chick showed considerable hypertrophy, it did not attain the weight of the normal pair of testes in the control (Breneman, 1950). Greater hypertrophy was shown by the remaining ovary in the unilaterally spayed rat, in which it was found that the remaining ovary in the mature animal was 100 per cent or more larger than the normal control ovary of the same age (Arai, 1921). Heller and Jungck (1947) found that the size of the spleen-grafted ovary in the rat was roughly 3 times that of the normal ovary. Table 1 shows that the single hypertrophied graft in the mice of series B averaged 6 times the weight of the normal ovary. In spite of the individual variation in these graft weights, the difference between their weight and that of the pair of control ovaries was significant at the 1 per cent level. The range

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FIG. 1. 100X Uterus of graft-bearing mouse (No. 211). The average uterus-vagina weight of animals in this group was 98.6 mg.

FIG. 2. 480X Same uterus as shown in Figure 1. The stromal nuclei are less fusiform in shape and less solidly staining than those of Figure 4 even though they are not as smoothly round and vesicular as those of Figure 5.

FIG. 3. 100X Uterus of one of the spayed animals. The average uterus-vagina weight for animals in this group was 46.5 mg.

FIG. 4. 480X Same uterus as in Figure 3. Note the solidly staining, closely packed fusiform-shaped nuclei. These are typical of the castrate uterus.

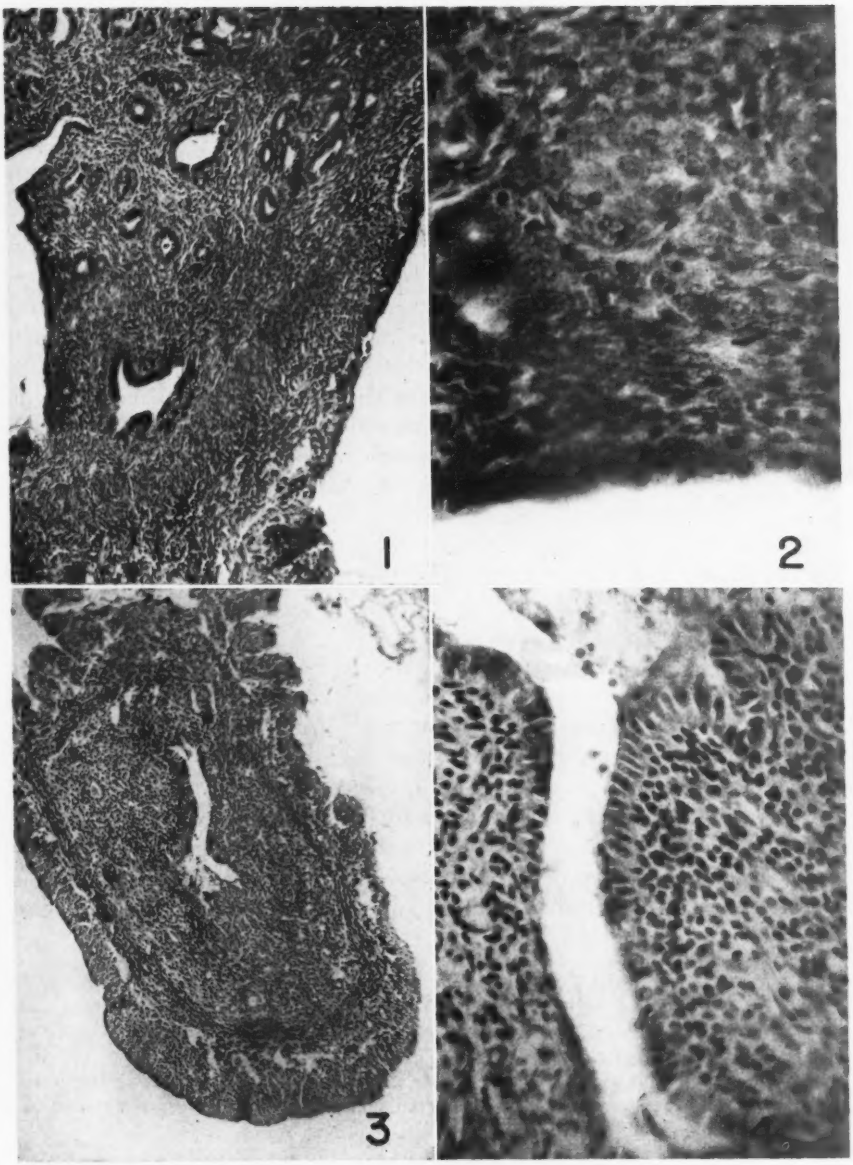

FIG. 1–4

in size was 0.29 to 5.59 mg. per gram of body weight, except for one graft (mouse number 210) weighing 4.723 grams, or 142.68 mg. per gram body weight. The data from this animal were not included in the averages. To the extent that ovarian weight is an index of ovarian activity, the single grafted ovary is indicated to be more active than the remaining gonad of the unilateral castrate.

Of the 14 average or larger than average uteri of series B, 12 were carried by animals having a below-average sized graft, while only 2 were accompanied by an above-average graft. Of the 19 mice with below-average sized uteri, 9 accompanied below-average grafts while 10 were with above-average grafts. There was no positive correlation between the large uterus and large graft; indeed, the animals carrying the above-average grafts seemed to have the smaller uteri and this suggestion of negative correlation was also found with the animals having the smaller grafts. Mouse number 210 seems to add to this, although this will be mentioned later. While the graft of mouse 210 was 84 times larger than average, the uterus was only 3 times as large. From this, it seems that one or both of two conclusions are in order: (1) either the size of the graft has little to do with the amount of its secretion, and/or (2) the amount (or percentage) of hormone removed by the liver varies greatly from animal to animal. The former possibility seems more likely because of the great variation in amount of follicular and luteal material found in the grafts.

Incomplete hepatic inactivation of the ovarian graft secretion was also indicated by microscopic observation. All of the five normal uteri observed showed oval shaped enlarged stromal nuclei with prominent nucleoli and finely granular chromatin (Figure 5), as described by Hooker and Forbes (1947). Of the 5 graft animal uteri, 4 showed a tendency toward this same condition, thus suggesting a mildly positive progesterone response. Comparison of Figures 1 and 3 shows very little difference in the overall appearance of the graft and castrate uteri, but comparison of Figures 2, 4 and 5 shows that while the graft nuclei are not smoothly rounded and vesicular

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FIG. 5. 480X Uterus of a normal control mouse (No. 5). The effect of estrogen and progesterone is seen in the wide spacing of the nuclei and the clearly visible nucleoli and finely granular chromatin. The average uterus-vagina weight of animals in this group was 190.6 mg.

FIG. 6. 480X Vagina of graft-bearing mouse (No. 211) showing mucification attributed to the action of progesterone. This is considered another indication that the secretions of the graft are incompletely removed by the liver.

FIG. 7. 100X Ovary of normal control mouse (No. 5).

FIG. 8. 100X Ovarian graft (mouse No. 211) showing developing follicles and luteal-like tissue. This represents a type of graft which resembles the normal ovary more closely than do many of the grafts.

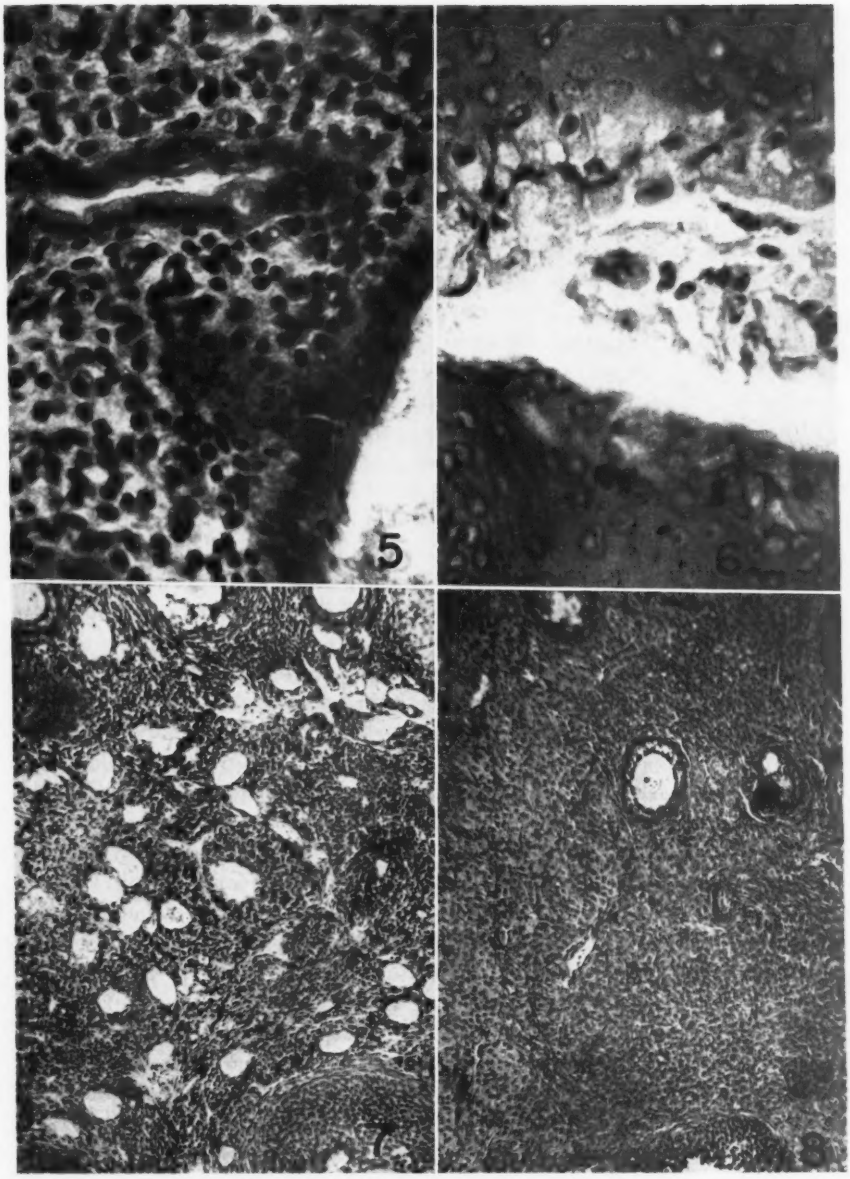

FIG. 5–8

like those of the normal controls, neither are they solidly staining and as fusiform in shape as are those of the castrates. Figure 2 shows a representative graft uterus; although some uteri show more of what is interpreted as a progesterone response, some show less. The uterus of Figure 5, on the other hand, shows more of the progesterone type of response than does that of many normals.

The vaginae of the graft-bearing animals show some effect of active ovarian hormone. Of the 5 examined, 3 showed mucification and some cornification, while the other two showed neither mucification nor cornification. Figure 6 shows the amount of mucification in graft mouse number 211. This is considered as further evidence for the presence of progesterone in the systemic circulation. Of 7 normal animals, 4 showed some cornification, but only 1 showed even a questionable amount of mucification. The number of animals showing cornification in this small sample of normals is of no significance since it merely indicates the part of the cycle that the animals happened to be in at the time of sacrifice. Figures 7 and 8 show a low power field of the normal ovary and of a graft. Both show developing follicles and luteal or luteal-like tissue. The most apparent difference is in the greater vascularity of the normal ovary; however, this is not intended to suggest that the vascularization of the graft is not adequate to meet the needs of the graft. The vascular pattern may have changed in response to the new conditions imposed upon the tissue.

Although the structure and organization of the normal ovaries vary within rather narrow limits, the grafts show a great variation. Some grafts show luteal bodies and several normal appearing follicles. Others show only a few small normal follicles, while the volume of the graft is made up of large irregularly shaped follicles either lying closely packed side by side or separated by the luteal-like tissue. Many grafts contain considerable luteal-like material which does not seem to be organized into "luteal bodies." Some of the large, irregularly shaped follicles may contain a small amount of blood and a few may be filled with it. This type of hemorrhage apparently follows degenerative changes and does not have the same significance as that found in the normal hemorrhagic follicle. An extreme case of what was considered "hemorrhagic degeneration" was found in the graft of mouse number 210. No nonhemorrhagic follicles were found and nearly the entire graft appeared to be made up of blood and blood degeneration products. The fact that the uterus was much heavier than the average, must however, indicate rather recent secretory activity of the graft.

It should be emphasized that information gained by other methods does not suggest any such imcompleteness of ovarian hormone inactivation as does the evidence presented here. The vaginal smear record of many graft-bearing mice observed in these groups as well as in others studied

shows that only occasionally does enough estrogen get through the liver to affect the vaginal smear reading. Very seldom is a cornified smear seen. Perhaps this in some cases is due to the masking of cornification by mucification. Casual autopsy observations suggest that the uteri of graft-bearing mice very closely resemble, because of their blanched appearance, those of the castrates rather than those of the normals.

No effort was made to determine the mechanism responsible for the incomplete hepatic removal of ovarian hormone described here but 3 possibilities may be mentioned: First, the liver may not act upon the total amount of estrogen and progesterone coming to it from the ovarian graft and some of the hormone may pass into the systemic circulation in its original active form. Secondly, some of the hormonal products derived during the process of hepatic inactivation may retain a certain amount of their activity as they enter the systemic circulation; and thirdly, the anastomoses between the portal and systemic veins may be large enough to allow some hormone to bypass the liver.

SUMMARY

The weights of the uterus and vagina of (1) spayed mice, of (2) spayed mice bearing an autoplastically grafted ovary in the spleen, and (3) of unoperated animals were compared. The organs of graft-bearing mice weighed significantly less than those of the controls; the organs of the castrates were in turn significantly lighter in weight than those of the graft-bearing mice. Histological examination of the uterus and vagina of the graft-bearing mice support the conclusion that sex hormone produced by an ovary situated in the spleen is not totally removed by the liver.

The average graft weight was 6 times that of a control ovary. A suggestion of negative correlation is seen between the weights of the grafts and the corresponding uteri.

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REESTABLISHMENT OF FUNCTION IN TRANSPLANTED THYROID GLANDS OF MICE*

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INVESTIGATION of the problem of transplantation of endocrine glands has proceeded almost entirely from the standpoint of histology and morphology, and up to the present time relatively little information has been obtained concerning the physiology of endocrine transplants. Some conclusions as to the functional condition of such implants have been drawn from observations on the varying degrees of success of autoplastic, syngenesial, and heteroplastic transplants. Additional information has been provided by observed differences in the ability of a graft to "take" depending on the presence or absence of the host's own gland. Evidence of this type is somewhat indirect, however, since it is based on interpretation of purely morphological data. In the case of the thyroid gland, a direct method has been developed for studying the functional properties of individual thyroid follicles by radioautography with I^{131}. It seemed to be of considerable interest, therefore, to apply this technique to the study of transplantation of the thyroid gland. Experiments have been carried out to analyze the structural and functional changes occurring in syngenesial transplants of thyroid tissue in the mouse. The results of this analysis are summarized in the present communication.

PROCEDURE

The animals used as hosts were 33 CFl male mice, approximately one month old at the time of the experiment. Grafts of additional thyroid lobes were made from donors of the same strain, sex, and age as the hosts. The hosts' own thyroids were left undisturbed. In each instance, one lobe of thyroid was placed in the end of a large bore trocar, and implanted under the skin of the top of the head of the host. It may be noted that this site of implantation was chosen because of the ease with which the graft could subsequently be recovered. Six different groups of about six mice each were sacrificed and the grafts recovered at the following intervals after transplantation: 6 hours, 24 hours, 3 days, 10 days, 21 days, and 40 days. In most experiments each animal was injected subcutaneously with 15μc. of I^{131} 24 hours before it was sacrificed. In the case of the 6-hour hosts, however, radio-iodine was administered at the same time that the implantation was

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made. Immediately after the animals had been sacrificed, the grafts were recovered and fixed in Bouin's solution. The tissue was then embedded in paraffin and sectioned at 10μ. Representative sections were mounted on slides, covered with a thin film of collodion, and placed in contact with no-screen x-ray film for 24 hours. After the sections had been stained with hematoxylin and eosin, a comparison was made of the histology and the relative uptake of radio-iodine by individual follicles as indicated by the radioautographs.

The diet fed to all mice was Purina Lab Chow. The animals were maintained under conditions which provided constancy of temperature ($\pm 1°$ C.) and humidity ($\pm 4\%$).

RESULTS

Thyroid implants underwent rapid necrosis, which was already marked within 6 hours after transplantation. The process of necrosis progressed up to 24 hours, and some evidence of repair was seen at 3 days. Ten days after transplantation a definite increase in the amount of typical thyroid tissue was observed. During this period, alterations in the capacity for iodine accumulation of the transplant, as indicated by radioautography, did not parallel the morphological changes noted. An approximately normal condition, as judged by the histological picture and the radioautographs, was

TABLE 1. SUMMARY OF HISTOLOGICAL AND RADIOAUTOGRAPHIC DATA

	Time					
	6 hours	24 hours	3 days	10 days	21 days	40 days
Number of animals	4	3	5	6	4	6
Intensity of autographs	strong	quite strong	very weak	strong	strong	very strong
Leucocytic infiltration	very marked	marked	marked	slight	slight	absent
Vascular connections	not evident	not evident	few and small	numerous	similar to normal	similar to normal
Epithelial condition	pycnotic	mostly pycnotic	some pycnotic some normal	mostly normal some pycnotic	normal	normal

reestablished within 21 to 40 days after the graft had been made. Observations on the condition of the transplants at the various stages which were studied are described in the following paragraphs, and summarized briefly in Table 1. Some of the findings are illustrated in Figures 1 to 12.

6-hour transplants: The transplanted tissue was almost entirely dead in the center, where no living follicles were found, and the tissue was heavily infiltrated with leucocytes. The superficial follicles were in various stages of pycnosis, and in many cases the colloid was dense and very eosinophilic. In a few thyroid follicles, the epithelium was less pycnotic, and the colloid

was somewhat vacuolated (Fig. 7). The capillaries present had the swollen epithelium indicative of degeneration. Occasional clumps of apparently healthy epithelial cells were found, but they were no longer in follicular arrangement.

A careful follicle-by-follicle comparison of the radioautographs and corresponding thyroid sections revealed that all autographs were made by the colloid of colloid-containing follicles. Some colloid-containing follicles did not give autographs, however. Although there was no constant histological difference between those follicles which did autograph and those which did not, it was noted frequently that the largest follicles did not autograph, while very small ones with small amounts of colloid did. In general, the autographs were strong and were limited to the periphery of the implant.

24-hour transplants: There was a greater proportion of dead tissue than at 6 hours, but the epithelium of surviving follicles was more cuboidal and less pycnotic. Relatively fewer leucocytes were present, although many follicles were still undergoing necrotic changes, and had leucocytes and macrophages in their colloid (Fig. 8). The capillary endothelium was still swollen. Numerous "ghost follicles," basophilic granular remnants of colloid with a hardly recognizable border of formerly cellular material, formed a large part of the medulla (Fig. 9).

As in the 6-hour transplants, all the autographs were made by recognizable colloid. There was very little correlation between the capacity to give a radioautograph and the histological appearance of a given follicle, except that, as noted previously, the small follicles with relatively little colloid were most likely to produce autographs. "Ghost follicles" did not autograph. Autographs were, in general, weaker than at 6 hours, and follicles giving autographs were still limited to the cortex.

3-day transplants: These resembled implants of 24 hours. There were a number of surviving follicles at the periphery, and some of these looked quite normal. Also at the periphery were many non-follicular cellular groups that appeared to consist of thyroid cells. In the center of most of the sections was a granular area containing very little cellular material other than loose fibrous tissue. Examination of the capsule of the transplant revealed a few healthy blood vessels of small caliber.

Very few large follicles yielded radioautographs. The great majority of the autographs were made by small follicles with comparatively little colloid. In some follicles the epithelium appeared to be regaining its normal structure but the colloid was full of leucocytes. Such follicles, in general, gave no autograph. A number of small reorganizing follicles were present. These contained very little colloid, and often, though not always, gave autographs. In general, the autographs at this time were limited to the

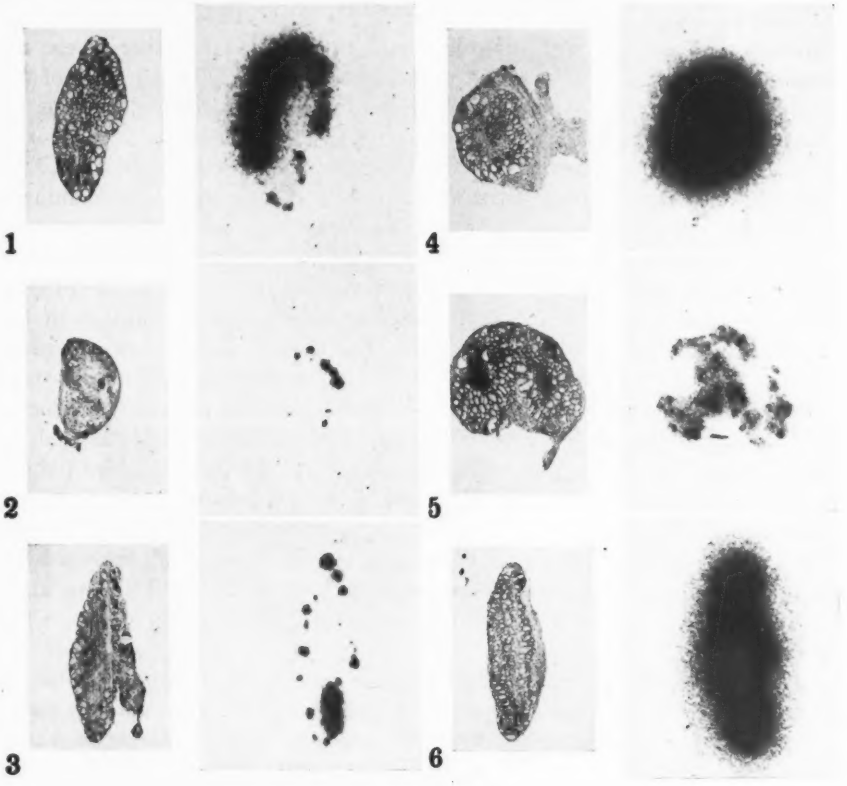

PLATE I. LOW POWER.

EXPLANATION OF FIGURES

FIG. 1. Section and radioautograph of transplant of 6 hours.
FIG. 2. Section and radioautograph of transplant of 24 hours.
FIG. 3. Section and radioautograph of transplant of 3 days.
FIG. 4. Section and radioautograph of transplant of 10 days.
FIG. 5. Section and radioautograph of transplant of 21 days.
FIG. 6. Section and radioautograph of transplant of 40 days.

cortex. The autographs were extremely weak, a finding which indicated that there was relatively little accumulation of organic iodine.

10-day transplants: As compared with the 3-day transplants, a larger number of surviving follicles, predominantly normal in appearance, was present at 10 days. In 4 of the 6 cases, the fibrous center was reduced in size, and relatively few leucocytes were present. The cortex contained a number of follicles with very large light cells and with little or no lumen.

In the case of 2 animals the implant had a large center of loose fibrous, almost non-cellular, structure. In these two transplants there were numerous pycnotic follicles, and a capsule had formed (Fig. 10). All of the follicles autographed, with the exception of a few very large ones. Thus the transplants resembled normally situated thyroid tissue in mice of this strain. One of the implants contained a nodule of metaplastic tissue in the medulla. The nodule consisted of comparatively large cuboidal or columnar cells with little or no colloid (Fig. 11), and may have been ultimobranchial tissue (Gorbman). As a whole, the autographs were strong and showed a fairly even distribution of I^{131} throughout the follicular areas of the implant.

21-day transplants: Some variation was noted in the condition of the transplants in this group of 4 animals. The graft in one mouse was practically normal in structure, although in some places non-follicular groups of thyroid cells were present. These cell groups seemed to be in the process of forming small follicles. The blood supply appeared to be established. In two other animals, the grafts still showed a considerable amount of pycnosis. The centers of the transplants were fibrous, and a number of macrophages and leucocytes were present. The cortex consisted largely of epithelial cells, however, and most of these cells were in follicular arrangement (Fig. 12). There were many more non-follicular thyroid cell groups present in the fourth graft, which may represent a slightly earlier phase in the process of recovery.

In this instance again, all the autographs were made by follicles containing colloid, although some apparently similar follicles produced no autographs. On the extreme periphery of the implants were some follicles whose epithelium appeared healthy, but which contained no colloid. These did not autograph. In general, the autographs at this stage were strong and quite similar to autographs from normal thyroid tissue.

40-day transplants: These grafts consisted of essentially normal-looking

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EXPLANATION OF FIGURES

FIG. 7. Surviving epithelium of 6-hour transplant. All the follicles but the one at lower right are able to metabolize iodine.

FIG. 8. Necrotic epithelium of 24-hour transplant. All the follicles pictured except the one at lower left are able to metabolize iodine.

FIG. 9. "Ghost follicles" or granular colloid remnants; the single large follicle at lower left did not yield a radioautograph.

FIG. 10. A small portion of the fibrous capsule is visible at lower right. The large follicles shown do not yield autographs.

FIG. 11. Nodule of metaplastic tissue from center of a 10-day implant. This area did not autograph.

FIG. 12. Normal-appearing thyroid tissue; all the follicles showing are able to metabolize iodine.

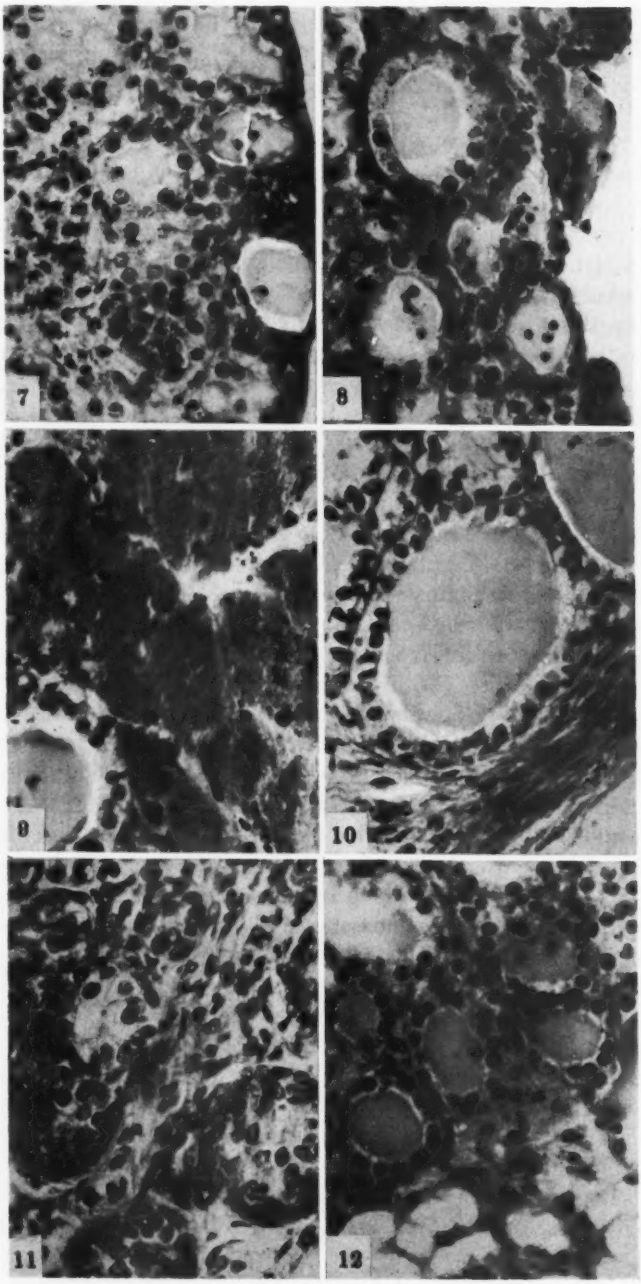

PLATE II. High Power.

tissue. Most had fibrous centers, but a thick follicular cortex was present in all cases. The autographs, as well as the histological appearance, were hardly distinguishable from normal.

DISCUSSION

In general, the cycle of structural alterations seen in the thyroid transplants of these experiments agrees with the earlier descriptions of Loeb. We have noted that medullary degenerative changes and leucocytic infiltration in the grafts are extreme six hours after transplantation. At this early interval, to judge from the necrotic condition of all vascular channels and cells within the tissue mass, any metabolic exchanges were probably limited to the surface of the transplant. In thyroids which remained in the host 1 to 3 days, the proportion of surviving, though often still pycnotic, cells became progressively larger. The leucocytic and macrophagic reaction gradually subsided, and was followed by fibrous replacement of medullary follicular tissue. Vascularization and cellular recovery, which was slow in the 1 and 3 day stages was accelerated after 3 days. At ten days the fibrous core of the transplant was almost free of leucocytes. Except for the presence of a reduced fibrous core, the 21 and 40 day stages were essentially indistinguishable from normal thyroid.

It is remarkable that strong radioautographs were obtained from thyroid grafts removed after a residence of only six hours in the subcutaneous tissue of the host. Moreover, in these instances the grafts were exposed to radioactive iodide for the relatively brief period of six hours. It should be borne in mind that, with the radioautographic technique used, only organic protein-bound iodine is detectable (Leblond). Therefore, in the six-hour period, surviving parts of the thyroid graft retain the ability to take up iodide, bind it organically, and accumulate it within the colloid in an apparently normal manner in spite of the very limited or absent vascularity and the simultaneous occurrence of active degenerative changes. A puzzling aspect of this phenomenon is its discontinuity. Adjacent follicles can differ in capacity to produce autographs, even though they appear to be similar in morphology and position in relation to the surface of the graft. Furthermore, follicles can autograph even though they contain a large proportion of pycnotic cells in their epithelia, and have many leucocytes in their colloid. Thus it was impossible to establish morphological criteria to distinguish transplanted thyroid cells which are able to metabolize iodide from those which cannot. This conclusion was drawn from a study of different follicles in the same graft, as well as from observations on follicles in grafts of varying lengths of residence in their hosts. The 1-day and 3-day grafts, for example, autographed more weakly than grafts of longer or shorter residence. Yet such thyroids contained a larger propor-

tion of epithelial cells of "normal" morphology than did the 6-hour grafts which autographed strongly. The 3-day implant, moreover, which gave the weakest autographs of all, was well vascularized as compared with the 6-hour and 1-day transplants.

In a few instances the failure of a large colloid-containing follicle to produce a radioautograph could be correlated with a characteristic histological appearance. Follicles with an irregularly alternating squamous and cuboidal epithelium, sometimes ciliated, and with a characteristic granular or "frothy" colloid are found in normal thyroids and can be considered of probable ultimobranchial origin (Gorbman). These do not yield an autograph in normally situated thyroids, and they did not in these experiments. It may be noted further that, although colloid surrounded by pycnotic cells often did accumulate radio-iodine, colloid completely surrounded by necrotic cells never did. Such colloid usually had lost the normal eosinophilia and had become chromophobic or slightly basophilic.

SUMMARY

The method of radioautography has been used to study the establishment of function in syngenesially transplanted mouse thyroids. The autographs obtained indicated that superficial thyroid follicles in the implant were capable of metabolizing iodine, in seemingly normal fashion, even when medullary necrosis was at a peak and before vascular connections between the host and graft had been established. One to three or more days after transplantation, when cellular recovery was occurring and vascular connections were being established, the function of the graft, as measured by iodine accumulation, was at the lowest level. Radioautographs produced by thyroid transplants of more than ten days residence in the host were essentially normal.

Under the conditions of the experiment, all autographs were made by radio-iodine accumulated in the colloid of the graft follicles. Not all colloid containing follicles autographed, however, even though they were morphologically indistinguishable from neighboring follicles that did.

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THE EFFECT OF ADRENAL CORTICAL EXTRACT, 11-DESOXY-17-HYDROXYCORTICOSTERONE ACETATE (COMPOUND S) AND 11-DEHYDROCORTICOSTERONE ACETATE (COMPOUND A) ON THE CARDIOVASCULAR-RENAL ACTION OF DESOXYCORTICOSTERONE ACETATE

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IT HAS been shown that the pressor effect of desoxycorticosterone acetate (DCA) in the rat is characterised by the association of a rise in blood pressure with an increase in heart weight (Friedman, Polley and Friedman, 1948). This effect can be produced by a small amount of the steroid in the intact animal even in the absence of supplementary salt. Similarly, the interference with renal function which follows the administration of DCA is constantly accompanied by a parallel increase in renal mass (Friedman and Friedman, 1949a). Based on these observations, we have recently set up a qualitative screening test in order to assess various compounds for their ability to inhibit the cardiovascular-renal effects of this synthetic steroid (Friedman and Friedman, 1950). This search for anti-DCA compounds was stimulated by the obvious although incompletely understood importance of the adrenal cortex in the pathogenesis of certain forms of hypertensive disease in man. Progesterone, testosterone, pregnenolone, estradiol, hyaluronidase, acetoxy-pregnenolone, "saturated 12-keto DCA" and, in later work, cortisone (Friedman, Friedman and Nakashima, 1950) were all incapable of inhibiting these effects of DCA. In marked contrast, Lipo-Adrenal Cortex (Upjohn), administered in sufficient amount, did oppose the DCA action.

The present communication is concerned with further tests of the one positive substance thus far observed, Lipo-Adrenal Cortex, as well as with the results with Compound S and Compound A.

EXPERIMENTAL

Methods: The screening procedure has already been described (Friedman and Friedman, 1950) and requires no detailed comment except insofar as a few minor modifications have been introduced. In general, male albino rats, closely matched at approximately 70

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grams in weight, are given free access to food and tap water. The first group of 8 animals serves as untreated control, while the second group of 8 animals receives subcutaneous implants of DCA (Schering) pellets. Each pellet is one-fourth (in former experiments one-third) of a 75 mg. Cortate pellet. Two such pellets are implanted on the first day of the experiment and one every four days thereafter to a total of 5 implants in two weeks. The method aims at providing a continuously increasing dose of DCA. The test group, of similar size, receives the same amount of DCA as that given to group 2, together with the compound being tested for anti-DCA activity. A fourth group to control the effects of the test substance itself is carried where possible.

Blood pressure is determined daily by the tail cuff method using ether anesthesia (Friedman and Friedman, 1949b) beginning on the 13th day and continuing through at least 6 determinations to termination.

At the end of the experiment the animals are weighed and killed, and hearts and kidneys are removed and fixed. The weight of these organs is expressed per unit of surface area. In the experiments here reported, the findings, as before, are presented as percentage deviation of the average values from the simultaneously determined control average. The blood pressure values used in the presentation represent the average of the last three successive determinations. It should be noted that as a result both of the brief duration of treatment and the use of ether the average elevation of blood pressure at termination is essentially of a minimal nature.

Experiment 1

Lipo-adrenal cortex. This extract had already been screened by the standard procedure and had shown a positive anti-DCA effect. The present experiment was designed to amplify this finding and consequently was altered from the standard screening procedure to terminate in the fourth week rather than in the third as usual.

Lipo-Adrenal Cortex (Upjohn) was injected subcutaneously, 0.5 cc. on each of the first 3 days of the experiment, 1.0 cc. per day thereafter. No control group was provided for the oily base of the extract since we had previously shown this to be without effect (Friedman and Friedman, 1950). DCA was administered as in the standard screening procedure but an extra pellet was implanted on the 22nd day of the experiment. Blood pressure was determined on 8 successive days beginning on the 20th day.

The results of this experiment are shown graphically in Figure 1.

DCA administered in these amounts and for this length of time produced a significant degree of blood pressure elevation accompanied as usual by an increase in heart weight. In marked contrast, Lipo-Adrenal Cortex given in these relatively large doses completely and effectively inhibited the rise in blood pressure and increase in heart weight. The enlargement of the kidney, which in our view reflects the rôle of this organ in the active metabolism of DCA, was not affected by the adrenal extract. It should also be noted that the extract caused an inhibition of body growth.

These findings confirm our previous report of the anti-DCA effect of this potent adrenal cortical extract. Because of the relative importance of

the blood pressure findings, Table 1 presents the day by day blood pressure averages in this experiment.

Experiment 2

Compound S (11-desoxy-17-hydroxycorticosterone actate): In this experiment, the test group of 8 animals received Compound S in addition to

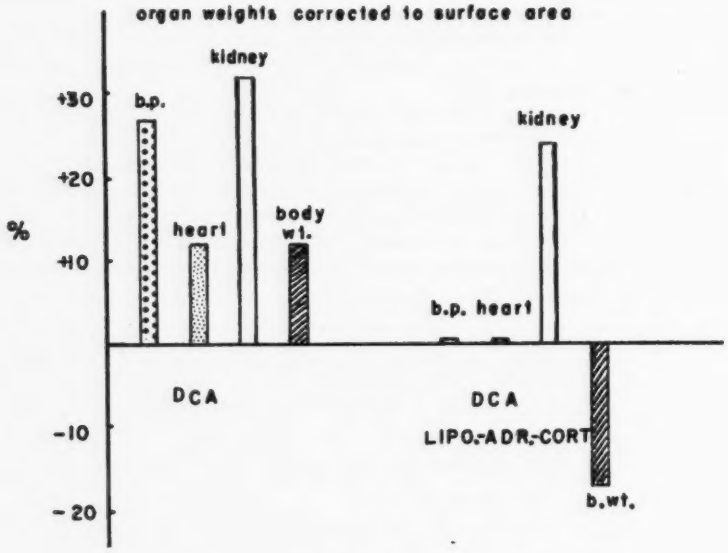

FIG. 1

DCA. Compound S was injected subcutaneously as a saline suspension of microcrystals with added suspending agents and 1.5% benzyl alcohol as preservative (Upjohn). Each cc. of the preparation contained 25 mg. of Compound S. Each animal received 0.05 cc. per day (1.25 mg.) for the first 12 days of the experiment and 0.1 cc. per day (2.5 mg) for the last 8 days.

TABLE 1

Blood pressure mm. Hg	Control	DCA	DCA+lipo-adrenal cortex
Day 20	117	134	122
21	112	130	116
22	109	132	126
23	115	146	128
24	121	146	136
26	116	154	115
27	122	149	111
28	114	144	121

An extra group of 8 animals receiving Compound S alone in identical amounts was carried in this experiment. The experiment was terminated on the 21st day.

The results are shown in Figure 2. Clearly, Compound S administered alone in these doses did not produce any significant effect. When given with DCA, the characteristic effects of DCA were in no significant way altered. It appears, therefore, that Compound S is to be added to the list of compounds which are incapable of inhibiting the cardiovascular-renal effects of DCA.

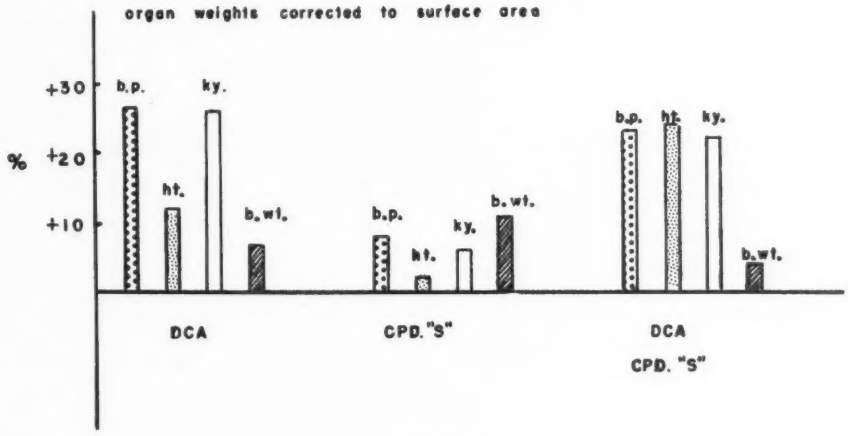

FIG. 2

Experiment 3

Compound A (11-dehydrocorticosterone acetate): In this experiment the test group of 8 animals received Compound A in addition to DCA. Compound A was injected subcutaneously as a saline suspension of microcrystals with added suspending agents and 1.5% benzyl alcohol as preservative (Merck). Each cc. of the preparation contained 25 mg. of Compound A. Each animal received 0.08 cc. per day (2 mg.) throughout the experiment. An extra group of 8 animals receiving Compound A alone in identical amounts was carried. The experiment was terminated on the 21st day.

This experiment was unsatisfactory and would not be reported at this time were it not for the unavoidable difficulties of obtaining more material for repeating the work. For some as yet unidentified reason, a new batch of animals received for this work showed a high incidence of spontaneous hydronephrosis accompanied by a mild hypertension and cardiac hypertrophy. Since such affected animals had to be discounted in the analysis of results, we do not feel that our findings are supported by a large enough

series of animals. The results are thus considered as indicative rather than conclusive.

The findings are presented in Figure 3. Compound A alone caused a significant retardation in body growth, an effect which was much more marked when the steriod was given with DCA.

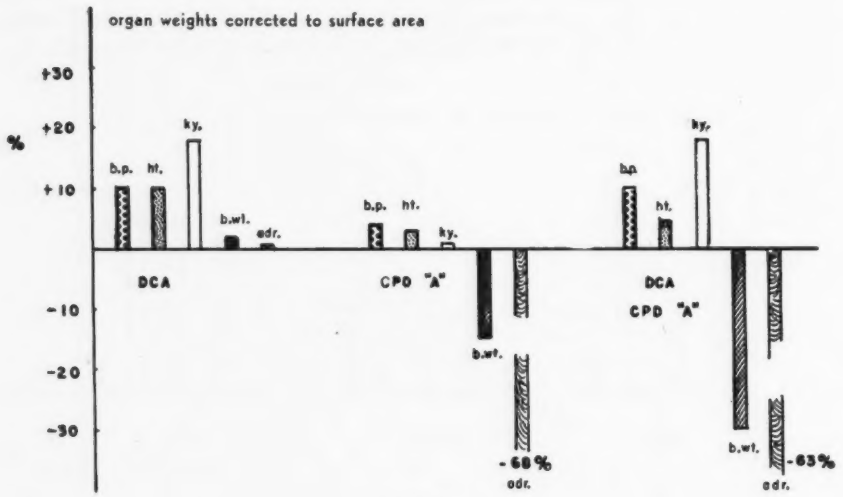

FIG. 3

Compound A given alone did not elevate the blood pressure or alter heart weight. When given with DCA it did not affect the hypertension caused by this steroid although in this case definite cardiac hypertrophy did not accompany the hypertension. This is probably referable to the marked inhibition of body growth observed when DCA and Compound A are given together.

The renal enlargement caused by DCA was not modified by Compound A, nor did the latter steroid affect kidney weight when given alone.

It would appear therefore that despite its limitations, this experiment indicates that Compound A itself has no definite cardiovascular-renal effects nor does it alter these effects as caused by DCA.

Because of the scarcity of basic data concerning Compound A, some additional observations were made. The steroid caused a marked decrease in the size of the adrenal gland. No clear cut effects on plasma sodium or potassium (Polley, 1948), as shown in Table 2, were observed. Histological examination of the kidneys of animals treated with Compound A showed no specific changes referable to the steroid.

DISCUSSION

The observation that Lipo-Adrenal Cortex inhibits the cardiovascular effects of DCA highlights the importance of dosage in assessing conflicting results. In our first work we failed to observe any effect with the extract, but had used only one-tenth cc. per day. Later, using a tenfold dose we observed the anti-DCA effect which we have amplified here in a longer experiment. These findings are in agreement with the observation in man (Pines *et al.*, 1948) and tend to emphasize the suggestion that the adrenal cortex may liberate anti-pressor material (Soffer, 1948).

TABLE 2

	Control	DCA	Compound A	DCA+compound A
Plasma Na mEq	146.2 (139.0–153.8)	152.0 (144.8–160.8)	147.4 (140.8–152.3)	151.5 (145.2–158.4)
Plasma K mEq	7.57 (6.97–8.90)	5.43 (2.72–7.05)	7.57 (6.28–9.15)	5.02 (3.46–7.05)

Our findings with regard to Compound S do not support Selye's observations that this compound has potent cardiovascular-renal effects (Selye, 1950). Selye, however, used somewhat larger doses of the steroid and in addition sensitized the rats by unilateral nephrectomy and the concomitant substitution of saline for drinking water. Such extreme conditions are difficult to assess in physiological terms. Certainly, the present experiment would not indicate any marked degree of activity for Compound S in the intact animal since a total of 35 mg. of this steroid given in the 20 days of the experiment failed to exert even the minimal cardiovascular-renal effects exhibited by approximately 10 mg of DCA (calculated on the basis of 100μg. of DCA absorbed per pellet per day).

Compound A likewise failed to exhibit any opposition to the effects of DCA in this test. This steroid did not exert any noteworthy effect when given alone other than a depression of growth and marked adrenal shrinking.

These results taken together tend to emphasize the positive anti-DCA effect of Lipo-Adrenal Cortex as well as the specificity of the screening test. Of the total of 11 substances tested so far, only this extract has shown anti-DCA effects.

SUMMARY

Lipo-Adrenal Cortex has again been shown to inhibit the elevation in blood pressure and increase in cardiac mass caused by DCA. The enlarge-

ment of the kidney produced by the latter steroid is not inhibited by this extract.

Compound S did not alter the cardiovascular-renal effects of DCA in the intact animal.

Compound A did not alter the cardiovascular-renal effects of DCA. It caused a marked interference with growth as well as striking reduction in adrenal size.

Acknowledgments

The DCA used in this work was generously supplied by the Schering Corporation. We are indebted to Dr. H. F. Hailman of the Upjohn Company for the Lipo-Adrenal Cortex and the Compound S; and to Dr. Augustus Gibson of Merck and Co. Inc. for the Compound A.

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INCREASE IN THE FORMATION AND SECRETION OF ACTH FOLLOWING ADRENALECTOMY[1]

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AFTER adrenalectomy, when it is to be presumed that there has occurred a marked reduction in circulating adrenocortical steroids, the concentration of ACTH in the blood has been shown to increase (Westman and Jacobsohn, 1944). This could be due to an increased formation and secretion of ACTH by the pituitary or a decreased removal rate of the hormone from the body.

This paper presents a method for the estimation of plasma ACTH after adrenalectomy, which when combined with estimation of pituitary ACTH content and the distribution and removal rate of the hormone, makes it possible to calculate its rate of formation and secretion by the pituitary. A more complete picture of ACTH turnover under these conditions may be thus furnished.

The results of assays intended to demonstrate the presence in the plasma of a substance with adrenocorticotropic activity have not been consistent. The conflicting results before 1948 could be ascribed to the use of insensitive test methods which employed animals with intact pituitaries. Jores (1935) deproteinized plasma and concentrated it by boiling. He assayed this material in immature mice and reported an increase in weight of the adrenals. Faber (1945) injected an equivalent of 10 cc. of plasma from adrenalectomized rats and was unable to demonstrate any increase in adrenal weight or histological changes in the adrenals of hypophysectomized rats.

In 1948 Sayers, *et al.* published a specific and sensitive method for ACTH assay. Using this method, Cooke *et al.* (1948) reported adrenocorticotrophic activity in normal human plasma concentrated by acetone precipitation. In 1949 Taylor *et al.* reported ACTH activity from untreated plasma of patients with adrenocortical insufficiency, but were unable to demonstrate this activity in plasma from normal subjects. Bornstein, *et al.* (1950) have reported ACTH activity in acid-acetone precipitates of normal hu-

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man plasma, and have also reported an increase in ACTH in the plasma of patients with Cushing's syndrome or congestive heart failure.

METHODS AND MATERIALS

Long-Evans male rats were used throughout this study. All animals were adrenalectomized at 40 days of age and blood was collected at intervals up to 4 weeks. The adrenalectomized rats were maintained on 1% NaCl in the drinking water. The operation was performed by the dorsal approach.

The adrenalectomized rats were anesthetized with ether. As much blood as possible was drawn from the inferior vena cava into a heparinized syringe. The plasma was separated by centrifugation. To the plasma was immediately added 4 times its volume of acid acetone (2.5 cc. of conc. HCl per 100 cc. of pure acetone) slowly and with stirring. The mixture was allowed to stand in the refrigerator for one hour, after which it was centrifuged and the supernatant decanted and saved. The precipitate was suspended in a volume of distilled water equal to the volume of plasma used and reprecipitated by the addition of 4 times this volume of acid acetone and again centrifuged. The two supernatants were added to 4 times their volume of pure acetone. The precipitate at this point was allowed to form by standing overnight in the refrigerator. This precipitate, which contained the active material, was separated by centrifugation, dissolved in saline, and adjusted to neutrality by the addition of a few drops of 1 N NaOH. This solution was then injected for assay.

An attempt was made to determine the completeness of recovery. A known amount of ACTH was added to plasma and shaken at room temperature for about 5 minutes. By extraction, all of the added ACTH was recovered.

The method for pituitary ACTH determination described by Burns *et al.* (1949) was followed. The anterior lobes were pooled from several animals, immediately frozen and lyophilized. An equivalent of 0.01 mg. of the dry pituitary powder was injected per 100 g. body weight of the assay animals.

As an assay for ACTH, the adrenal ascorbic acid depletion method of Sayers *et al.* (1948) was used. The assay animals were male Long-Evans rats, 40 days of age, weighing approximately 130 g. Twenty-four hours after hypophysectomy, the assay rats were anesthetized with sodium pentobarbital, the left adrenals removed, weighed and prepared for the analysis of ascorbic acid. The extract to be assayed was injected via the saphenous vein, 0.5 cc. per 100 g. body weight. One hour later the right adrenal was removed, weighed and prepared for ascorbic acid analysis. From six to ten animals were used in each assay. The difference between the concentration of ascorbic acid in the left and the right adrenal—the "depletion"—was calculated and expressed in mg. per 100 g. wet adrenal. This difference when compared to a standard chart represented the ACTH potency. All results giving a depletion of less than 25 mg. per 100 g. wet adrenal weight were considered negative.

An Armour Laboratories ACTH preparation standardized against their La-1-A was used as a reference standard. The content of ACTH in pituitary and body fluids of this study is therefore calculated and given as an equivalent to this standard. See Figure 1. The slope and sensitivity of this curve compares favorably to that obtained by Sayers *et al.* (1946) for another preparation standardized against La-1-A.

The possibility that adrenalectomy influences the disappearance rate of ACTH and its space dilution must be considered. A method used by Greenspan *et al.* (1950) for determination of the disappearance rate of ACTH was followed. One mg. of ACTH was

injected intravenously into normal male rats and into rats which had been adrenalectomized one hour or twenty-four hours previously. Untreated plasma from the injected animals was injected into the assay rats.

RESULTS

ACTH content in the pituitary and in the body fluids of normal rats: The average pituitary content of ACTH was found to be 58.3 microg. per dry gland from male Long-Evans rats weighing 140 g. In the body fluids of normal rats, all attempts to find ACTH failed. As much as 448 cc. of rat

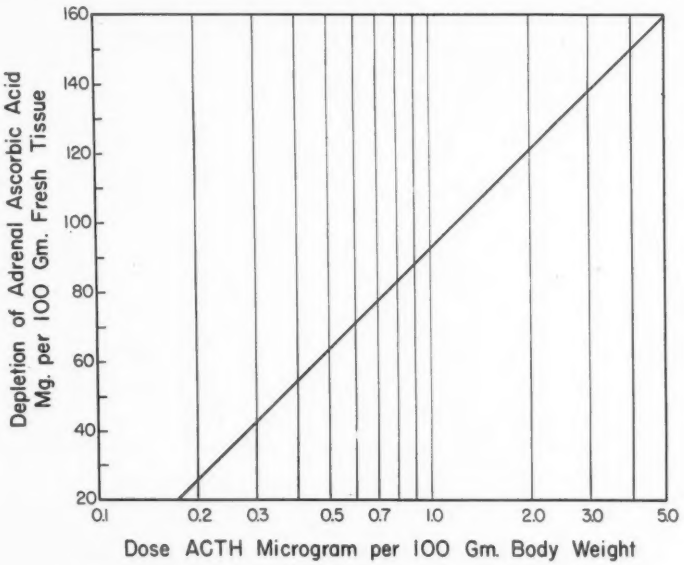

FIG. 1. Depletion of adrenal ascorbic acid following intravenous administration of ACTH.

plasma was precipitated without any trace of adrenocorticotrophic activity when tested with the adrenal ascorbic acid depletion method. A minimum response (25 mg. per 100 g. adrenal) with this amount of plasma (448 cc.) would have meant a concentration of 0.006 microg. per cc. plasma. Therefore, a negative response indicated that the level of ACTH in normal rat plasma is less than 0.006 microg. per cc. (Tables 1 and 2).

ACTH content in the pituitary and in the body fluids after adrenalectomy: Immediately after adrenalectomy there was a sharp drop in pituitary ACTH content. After 24 hours the pituitary ACTH content began to rise and eventually reached a level much above the normal. In the plasma after adrenalectomy there was an immediate rise in ACTH, which tended to level off after approximately 4 days (Tables 1 and 2 and Fig. 2).

TABLE 1. ACTH CONTENT OF PITUITARIES OF NORMAL AND ADRENALECTOMIZED RATS

Type of animal	Average wt. of rats	No. of pituitaries in sample	Average wt. of a single dry pituitary	Average value of adrenal ascorbic acid depletion†	ACTH per mg. dry pituitary	Average ACTH per mg. dry pituitary	Average ACTH per pituitary
	gm.		mg.	mg./100 g.	μg.‡	μg.‡	μg.‡
Normal							
41 days old:	133±3.4*	14		83± 4.8	80.0		
	131±2.7	9		82± 6.7	77.1		
	130±2.9	7		83± 5.5	80.1		
	137±4.0	7		80± 5.9	75.1		
	132±3.3	7	0.77	85± 7.6	85.1	75.8±3.3	58.3
	130±2.2	7		73± 4.8	63.0		
	129±2.9	7		70± 7.9	59.0		
	132±3.5	7		83± 9.8	80.1		
	129±3.1	7		89±12.8	93.0		
	146±3.0	7		74± 6.6	65.2		
47 days old:	174±3.7	8	1.10	74± 3.5	65.1	65.1	71.6
61 days old:	236±7.5	5	1.38	78± 7.0	71.1	71.1	98.0
Adrenalectomized							
1 hour	134±4.5	7	0.68	70± 9.6	59.0±14.0	67.1	45.6
	130±3.0	9		80± 6.7	75.1±12.5		
3 hours	133±2.5	7	0.73	63± 7.3	49.0± 8.5	44.0	32.1
	129±1.6	7		53± 8.3	39.0± 7.5		
6 hours	130±2.6	7		47± 7.3	33.5±12.0	30.7	23.3
	130±3.5	7	0.76	41± 6.4	29.0± 8.5		
	134±3.0	7		42± 6.3	29.5±14.0		
24 hours	130±2.2	7	0.75	28± 8.5	21.0± 7.5	22.8	17.1
	129±2.9	7		32± 4.9	24.5± 6.0		
72 hours	139±3.9	7	0.82	51± 7.2	36.5± 6.3	36.5	29.9
168 hours	174±4.1	7	1.03	104± 5.6	135.0±20.0	135.0	139.0
504 hours	220±4.0	10	1.19	107± 4.0	145.0±15.0	145.0	172.0

* Standard deviation.
† 9–10 animals in each assay. An equivalent of 0.01 mg. dry pituitary inj./100 gm. body wt. of assay animal.
‡ La-1-A equivalent.

TABLE 2. ACTH CONTENT OF RAT PLASMA AFTER ADRENALECTOMY

Time after adrenalectomy	Volume of plasma in sample	Final dilution of sample to be assayed	No. of animals in assay	Average value of adrenal ascorbic acid depletion	ACTH per cc. plasma	Total ACTH in body fluids
	cc.	cc.		mg./100 g.	μg.*	μg.*
0 min. (normal)	448	7	7	0	<0.006	<0.34
10 min.	132	6	6	25±10.8	0.018±.005	1.01± .546
1 hour	280	5	7	58 4.2	0.016±.001	0.90± .337
3 hours	251	7	11	87 4.6	0.050±.004	2.80±1.091
3 hours	213	7	7	42 10.4	0.020±.005	1.12± .582
6 hours	79	5	6	57 3.3	0.054±.004	3.02±1.164
6 hours	116	7	9	60 7.2	0.055±.010	3.08±1.438
10 hours	74	7	9	52 6.0	0.072±.011	4.03±1.791
16 hours	65	5	7	65 6.2	0.080±.012	4.48±1.984
20 hours	46	7	9	54 4.2	0.122±.012	6.83±2.754
30 hours	41	7	8	27 5.1	0.070±.009	4.06±1.676
40 hours	37	5	6	62 6.3	0.130±.021	7.45±3.300
50 hours	41	7	8	48 6.7	0.116±.020	6.97±2.995
69 hours	46	7	10	68 5.3	0.131±.021	8.19±1.310
144 hours	55	7	9	86 4.9	0.222±.029	15.20±5.330
1 week	60	7	10	99 5.4	0.280±.035	21.00±6.660
1 week	60	7	10	87 7.7	0.203±.027	15.20±4.890
2 weeks	69	7	10	84 9.3	0.167±.037	13.70±4.650
3 weeks	60	7	10	86 3.3	0.201±.016	19.20±4.385

* La-1-A equivalent.

The measured data for pituitary and body ACTH are shown in Figure 2 as a function of time. Plotted on the same graph is also the data of increased pituitary ACTH content in normal controls due to growth and the data for the assumed upper limit of normal body fluid ACTH.

Determination of the ACTH space of the body and biological half life of ACTH before and after adrenalectomy: The disappearance rate of ACTH injected intravenously into normal male rats, weighing 500 g. has been reported by Greenspan *et al.* (1950) and was confirmed in this study using normal rats, weighing 140 g. After adrenalectomy neither the ACTH space

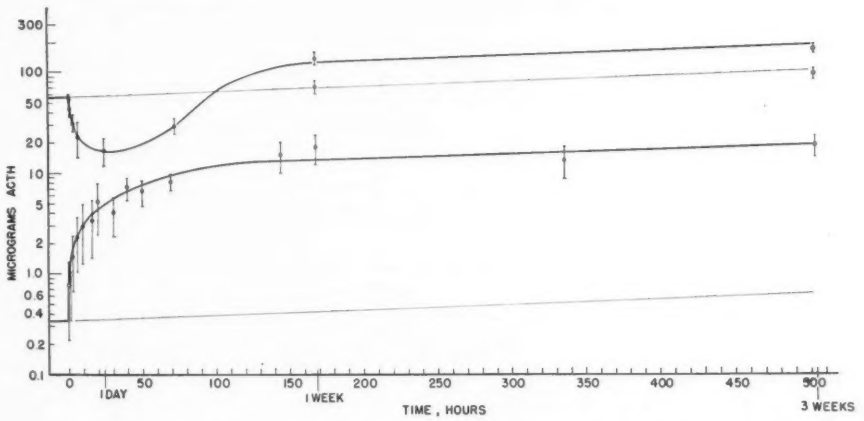

FIG. 2. Content of ACTH in pituitary (above) and body fluids (below) of adrenalectomized and normal rats; in each case the straight line represents the ACTH content in normal rats.

nor the disappearance rate of the ACTH from the plasma was significantly altered. In both studies great variations in the ACTH determinations were obtained, but the level of ACTH in the plasma appeared to fall in a logarithmic fashion against time. When extrapolated to zero time an average value of 6% of the injected ACTH was found in the total plasma. Assuming that this small concentration at zero time resulted from the dilution of ACTH, the ACTH space was calculated and was found to be 43% of the body mass. The biological half life of injected ACTH was not influenced by adrenalectomy. In normal and adrenalectomized rats it was found to be 4 to 10 minutes with an average value of approximately 5 minutes. We thus conclude that the biological half life of ACTH is 5 minutes and the ACTH space is 43% of the body mass and that these variables are not changed by adrenalectomy more than by a factor of two; they will be regarded as constant in the calculations to follow.

Analysis of the results

In order to make optimal use of the data it seemed worthwhile to analyze the data mathematically. On this basis, some conclusions were drawn that would have been difficult to reach without these analyses. In spite of the large fluctuation of the individual data, when they are regarded collectively, a fairly definite picture is obtained of the time sequence of events.

Based upon previous theories, a simple ACTH flow model (Fig. 3) was devised for visualizing the formation and secretion of this hormone. The

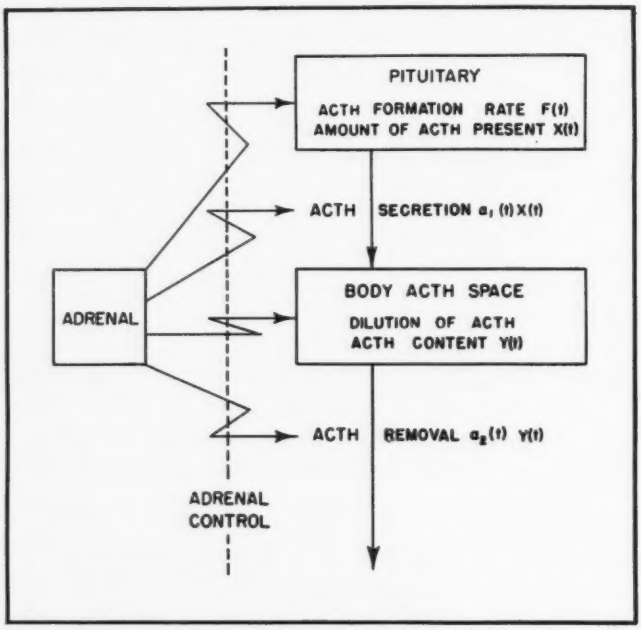

FIG. 3. ACTH flow model.

calculations were made with the assumption that such a model is substantially correct.

Interpretation is made difficult due to the large fluctuations in the data, and due to the fact that it was not possible to secure more than one set of data from the same animal.

As is shown in Figure 3, formation of ACTH is assumed to be entirely in the pituitary at the rate of $F(t)$ per hour, t representing time. The pituitary ACTH content is $X(t)$. The pituitary ACTH is secreted into the body fluids at a rate of $\alpha_1 X(t)$, where α is the *pituitary secretion coefficient.*

An important distinction is made between the terms *formation* and *secretion* of ACTH. The former refers to the amount of hormone actually synthesized in the pituitary, the latter to the amount actually transferred to the body fluids.

In the body fluids, the ACTH is distributed in an apparent volume (V), called ACTH space. The ACTH content in body fluids will be denoted by symbol $Y(t)$. ACTH disappears from the body at the rate of $\alpha_2 Y(t)$. The *disappearance constant*, α_2, is connected to the half time by removal T_2 by the relation:

$$\alpha_2 = \frac{\log 2}{T_2}.$$

Four possible processes may be conceived which could be fully or partially controlled by adrenal steroid secretion. These are:

a. ACTH formation $F(t)$ by the pituitary
b. Rate of secretion of ACTH from the pituitary
c. The ACTH space (V) in the body
d. Rate of ACTH disappearance $\alpha_2(t)$

Both the pituitary and body ACTH are functions of the above quantities.

In a steady state of ACTH balance, such as seems to exist in normal rats prior to adrenalectomy and in adrenalectomized rats three weeks after the operation, the ACTH balance may be expressed as:

$$\alpha_1 X = \alpha_2 Y = F. \qquad (1)$$

The meaning of equation (1) is that in a steady state ACTH secretion rate equals its formation rate.

In Table 3, measured values of X, Y and α_2 are given along with values of α_1 and F, calculated from (1).

TABLE 3. DATA EMPLOYED IN ESTIMATING THE TURNOVER OF ACTH IN RATS

	Normal	Normal	Adrenalectomized (3 weeks p.o.)
Age (days)	41	62	62
Body mass (g.)	132	231	220
Mass of dry pituitary (mg.)	0.76	1.3	1.2
X(Pituitary ACTH, μg.)	58	102	172
ACTH Space (cc.)	57	99	95
Plasma ACTH (μg./cc.)	≦0.006	≦0.006	0.2
Y(Body ACTH, μg.)	≦0.34	≦0.59	19
Biological half life of ACTH (min.)	5	5	5
α_2(Disappearance constant, /hr.)	8.3	8.3	8.3
α_1(Secretion coefficient, /hr.)	≦0.048	≦0.048	0.92
F(Pituitary ACTH formation rate, μg./hr.)	≦2.8	≦4.9	158
$\alpha_1 X$(Pituitary ACTH secretion, μg./hr.)	≦2.8	≦4.9	158

It will be seen from Table 3 that a normal animal's pituitary contains more than a hundred times as much ACTH as the total body fluids and that three weeks after adrenalectomy, when a new equilibrium is established, the pituitary ACTH content is one and one half times that found normally and the ACTH level of the plasma has increased at least 30 times. The formation and secretion rates of ACTH in the pituitary are calculated to have increased at least 30 times.

The rearrangement that occurs after adrenalectomy is clearly of a very complex nature, and is likely to follow higher orders of reaction rates. For this reason we assumed no more than that the law of conservation of mass holds in the process.

In the pituitary, on the basis of the scheme proposed in Figure 3, the balance between ACTH content $X(t)$, formation rate $F(t)$ and transfer to body fluids may be described as follows:

$$\frac{dX}{dt} = F - \alpha_1 X \tag{2}$$

while the ACTH balance for the body fluids, according to Figure 3 is

$$\frac{dY}{dt} = \alpha_1 X - \alpha_2 Y. \tag{3}$$

The experiments furnish $X(t)$ and $Y(t)$. $F(t)$ and $\alpha_1(t)$ are unknown. By adding (2) and (3) and rearranging, we obtain:

$$\frac{dX}{dt} + \frac{dY}{dt} + \alpha_2 Y = F. \tag{4}$$

Equation (4) gives the ACTH *formation* rate of the pituitary.

Expressing $\alpha_1 X$ from (2) or (3):

$$\alpha_1 X = \frac{dY}{dt} + \alpha_2 Y = F - \frac{dX}{dt}. \tag{5}$$

Equation (5) expresses the *secretion* rate of ACTH from the pituitary.

It seems convenient to approximate the measured functions $X(t)$ and $Y(t)$ in terms of exponential functions; this was done to simplify calculations and it does not imply that the functions describe some actual physical process.

One approximation for the body ACTH $Y(t)$ (lower portion of Figure 2) is given as follows:

$$Y(t) = 10.6(1 - e^{-0.015t}) + 2(1 - e^{-0.267t}) + 0.34 + 0.0156t. \quad [\mu g.]$$

The first two terms of $Y(t)$ refer to the gradual increase of ACTH in the body fluids due to adrenalectomy. The third term is the amount of

ACTH present at the time of adrenalectomy and the last term in the equation is a correction for growth of the animal during the experiment.

From $Y(t)$, using equation (5) the rate of secretion of ACTH from the pituitary may be calculated. The secretion is chiefly governed by the amount disappearing from the body $\alpha_2 Y$ and only in a negligible way by the amount of ACTH needed to build up the body level and the pituitary level of this hormone.

The ACTH secretion:

$$\frac{dY}{dt} + \alpha_2 Y = F - \frac{dX}{dt} = 16(1 - e^{-0.287t}) + 88(1 - e^{-0.015t}) + 2.8 + 0.13t \ [\mu g.]$$

The total formation rate $F(t)$ of ACTH in the pituitary is almost the same as the secretion, except for the first few hours, when the rate of change of pituitary ACTH content is comparable to the rate of formation. Figure 4 shows both the ACTH secretion rate and formation rate as a function

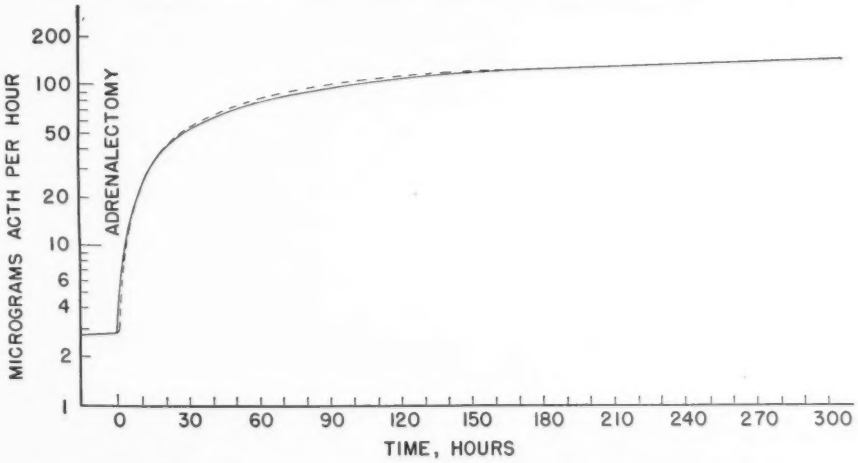

FIG. 4. ACTH formation (dotted line) and secretion (thick line) rates. The difference, which is exaggerated in the figure, is most significant in the first few hours.

of time. Draining of the stored ACTH from the pituitary allows a lag in the ACTH formation in the first few hours after adrenalectomy. The pituitary secretion coefficient α_1 may be calculated on the basis of equation (5):

$$\alpha_1 = \frac{\alpha_2 Y + \dfrac{dY}{dt}}{X}.$$

The values for this function are plotted in Figure 5; the change in ACTH

secretion with time is very marked, the peak secretion coefficient being about 75 ± 35 times the upper limit of normal. However, the magnitude of the standard error also shown on some points of the graph is considerable.

The two variables primarily affected by adrenalectomy are the pituitary ACTH formation rate and the ACTH secretion coefficient. To obtain complete solutions for the differential equations written, (2) and (3), one would

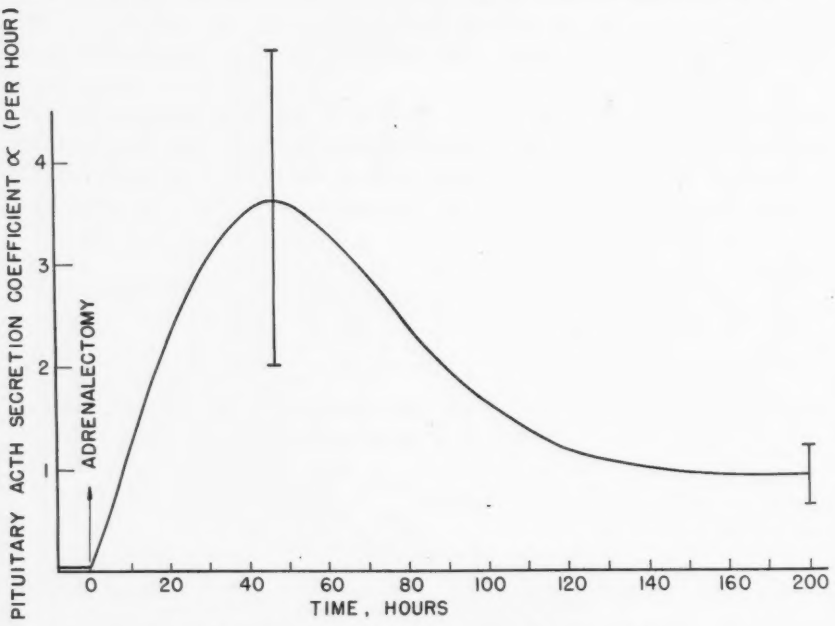

FIG. 5. ACTH secretion coefficient as a function of time.

want to make physical assumptions for laws governing the rate of change of these functions. However, the changes in the secretion coefficient, as Figure 5 shows, must be quite complex, and seem to depend on factors as yet unknown. The pituitary ACTH content reflects the changes in secretion and formation rate. It presents a picture of depletion and recovery to a somewhat higher than normal level, but stands in no direct relation to the greatly increased formation and secretion rates.

The above considerations were based on total amounts of ACTH in the body. Similar considerations hold for the ACTH concentration per mg. of tissue and for secretion per unit volume of the gland. As the data show, the ACTH content and pituitary mass are proportional.

DISCUSSION

Much evidence supports the concept that the level of adrenocortical hormones in the body fluids is a major regulator of ACTH secretion from the pituitary. It has been demonstrated that the weight of the adrenal gland (Tepperman *et al.*, 1943) and the concentration of ascorbic acid in the adrenal (Sayers *et al.*, 1946) are under the specific regulatory control of ACTH. Chronic administration of adrenal cortical extract (ACE) causes a decreased adrenocorticotrophic activity of the pituitary as shown by adrenal atrophy (Ingle *et al.*, 1938). The hypertrophy of the adrenal, which occurs after forced exercise does not occur when the animals are pre-treated with ACE (Ingle, 1938). Also the drop in adrenal ascorbic acid content, which occurs when the animals are exposed to cold, can be prevented by pre-treatment with ACE or cortical steroids (Sayers *et al.*, 1947). Therefore, it is generally assumed that the cortical hormones have an inhibitory effect on the secretion of ACTH from the pituitary.

This study has shown that the absence of the adrenal and, hence, presumably of the adrenal steroids from the circulation, is a stimulus to the pituitary secretion and formation of ACTH. If the level of cortical hormones in the body fluids is the only regulator of ACTH formation and secretion from the pituitary, it would be expected that ACTH secretion after adrenalectomy occurs at a maximal rate.

The concept that removal of an endocrine target organ, which is maintained by a trophic stimulus from the anterior hypophysis, induces hypersecretion of this trophic hormone, is based on experiments involving castration (Matsuyama, 1921), thyroidectomy (Van Dyke *et al.*, 1950) and adrenalectomy (Houssay *et al.*, 1944, Westman *et al.*, 1944). Following castration and thyroidectomy, there is an increase in the amount of gonadotrophins or thyrotrophic hormone in the pituitary, which is concordant with the demonstration here of a high ACTH content in the pituitary a few days after adrenalectomy. However, immediately after adrenalectomy there is a fall in the ACTH content of the pituitary. A similar fall has not been demonstrated in the pituitary content of the gonadotrophins and thyrotrophic hormone following castration or thyroidectomy.

The removal of the adrenal glands does not significantly alter the disappearance rate of ACTH in the body. The amount of ACTH utilized in the adrenal glands is apparently only a small part of the total ACTH present in the body. Richards *et al.* (1950) have shown that even in eviscerated rats the disappearance rate of ACTH is the same as in normal rats.

CONCLUSION

The adrenal gland through its formation of cortical steroids controls the formation and secretion of ACTH by the pituitary. Removal of the ad-

renals is reflected in an immediate and progressive increase in ACTH secretion. This causes the pituitary ACTH content to drop until the progressively increasing ACTH formation by the pituitary causes it to rise again. Adrenalectomy does not appreciably affect the ACTH space or the disappearance rate of ACTH.

Three weeks after adrenalectomy, a new approximately steady state is reached, characterized by a content of ACTH in the pituitary one and one-half times normal, an increased ACTH content in the body fluids at least 30 times that found normally, and an increased ACTH formation and secretion rate at least 30 times normal.

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THE PITUITARY GONADOTROPIC ACTIVITY OF STALK-SECTIONED MALE RATS[1]

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INTRODUCTION

THERE is general disagreement in the literature on the effect that infundibular stalk section has on the activity of the anterior lobe of the pituitary as expressed in changes of endocrine organs normally maintained by pituitary tropic hormones (see reviews by Harris, 1948; Friedgood, 1950). In previous communications (Greep and Barrnett, 1951; Barrnett and Greep, 1950) we have shown that panhypopituitarism, varying in severity, occurred in female rats after stalk section, as indicated by hypofunction of the ovaries, adrenals and thyroids. Fibrotic scarring and changes in cellular morphology appeared in the pituitary glands of these animals. The possibility was advanced that the endocrine hypofunction encountered in these experiments could be attributed to anemic infarction of the pituitary, since transection of the stalk interrupted the major blood supply and there was no subsequent regeneration of the stalk vessels.

The present work extends to the male rat studies on the effects of stalk section on the pituitaries and gonads already reported for the female. In contrast to reports on the female, relatively few observations are available in the literature concerning stalk section in males. In addition, experiments on castration which are known to influence both the structure and function of the anterior lobe, were combined with pituitary stalk section so as to afford additional information concerning the interrelationships which affect that organ. Finally histological observations of the pituitary gland were made at varying intervals after operation on the stalk, in order to establish more clearly the sequence of events leading to the fibrotic scarring previously reported by us.

METHODS

Male albino rats quartered in a constant temperature room and fed a diet of fortified ground dog chow were used as experimental animals. These rats were divided into 3 ex-

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perimental categories. The rats used in the first experiment weighed within 5 grams of each other and included two control and one experimental group. The latter group consisted of 16 animals which were stalk-sectioned according to the technique previously described (Greep and Barrnett, 1951). Seven normal control rats (*initial controls*) were killed with ether at the time of operation on the experimental animals. Six other normal controls (*conclusion controls*) were killed along with the stalk-sectioned rats on the thirty-fifth day of the experiment. At necropsy the weight of the body, testes, seminal vesicles, adrenals and thyroids of all animals was recorded. One testis of each stalk-sectioned rat was fixed in 10% formalin, imbedded in paraffin, sectioned at 7μ and stained with hematoxylin and eosin. The weight of the pituitary gland was also recorded for each control animal and some of the operated ones.

The heads of 8 stalk-sectioned rats were dissected with the aid of a low power microscope and gross details concerning the pituitary gland and the stalk were noted. These pituitaries were removed, weighed and fixed in Zenker's fluid. Paraffin sections cut at 7μ were stained by the Heidenhain azan method and studied microscopically.

The heads of the remaining 8 stalk-sectioned rats were fixed in 10% formalin after perfusion of the vascular system with a 50% aqueous solution of Higgins' waterproof India ink (Barrnett and Greep, 1951). Following subsequent decalcification in 5% trichloroacetic acid and celloidin imbedding, serial sections were cut, alternately at 100μ and 10μ in the sagittal plane, through the region of the pituitary gland, stalk and hypothalamus. The 10μ sections were stained with Heidenhain's azan, while the 100μ sections were left unstained. All were examined for completeness of stalk section, as well as for the continuity of the hypophyseal-portal blood vessels.

The second category consisted of 21 rats which were both stalk-sectioned and castrated, 14 rats which were only castrated, and 13 rats which were only stalk-sectioned. These animals were killed 75 days after operation and the body, thyroid and adrenal weights recorded. The testes and seminal vesicles of the rats stalk-sectioned only were weighed and the testes were prepared as above for microscopic examination. The weights of the seminal vesicles of the rats castrated only were also noted. The pituitary glands of all rats were examined *in situ*, then removed and weighed. Five pituitaries selected at random from each of the three operated groups were fixed in Zenker's fluid, sectioned at 7μ in paraffin, and stained by the Heidenhain azan technique. The pituitaries of the remaining animals were stored in acetone for assay of gonadotrophic content.

Assay of the 33 pituitaries was performed in the following manner: The acetone-dried weights were recorded. In the case of the castrated rats, only one-half of the anterior lobe was used in order to approximate the average weight of the pituitaries of these rats to each of the other two groups. The dried pituitaries were ground separately and suspended in 4 cc. aliquots of physiological saline. Each one of these 33 suspensions was injected subcutaneously into a 25-day-old female rat which had been hypophysectomized 2 days previously. An injection dose of half a cubic centimeter of the pituitary suspension was given on the afternoon of the first day, twice a day on the succeeding 3 days, and again on the morning of the fourth day. Six hours after the last injection, the 33 recipient rats, as well as 7 uninjected, hypophysectomized, recipient controls, were killed by ether. The weight of the ovaries, uterus and adrenals of these rats was recorded and compared.

In the final category, 16 adult male rats were used to study the changes leading to central fibrosis of the pituitary gland after stalk section. These animals were stalk-sectioned and were killed by ether in groups of four on the first, third, seventh and fifteenth postoperative day. At autopsy the heads of these rats were dissected and the pituitary glands examined, removed, fixed in Zenker's fluid and stained by the Heidenhain azan method.

RESULTS

I. *The effect of stalk section in male rats*

A. *Testes and seminal vesicles:* Testes of the stalk-sectioned rats retracted into the abdomen within a few days after the operation. The average weight of the testes of these animals killed 35 days postoperatively was 678 mgs. or about 23% of the average testes weight (2920 mgs.) of the conclusion controls (Table 1). In 12 of these operated rats the testes weighed less than 380 mgs. each, and in 4 between 900 and 1700 mgs. The seminal vesicles of the stalk-sectioned rats were also much smaller than

TABLE 1. ORGAN WEIGHTS OF STALK-SECTIONED, STALK-SECTIONED, UNCASTRATED AND UNOPERATED CONTROL MALE RATS

	Day sacrificed	No. rats	BW (gm.)	Testes (Mg.)	Seminal vesicles (Mg.)	Adrenals (Mg.)	Thyroids (Mg.)	Pituitaries (Mg.)
Initial controls	0	7	169.1	1920	235.1	28.9	10.4	5.2
Stalk-sectioned	35	16	181.0	678	104.0	17.9	9.3	3.2
Conclusion controls	35	6	271.6	2920	541.1	38.4	15.8	6.9
Castrated	75	14	327.0	—	23.9	44.5	17.2	13.3
Stalk-sectioned	75	13	232.4	437	83.8	17.8	11.8	4.1
Stalk-sectioned, castrated	75	21	242.8	—	—	21.8	12.1	5.0

those of control animals (Table 1). The 4 stalk-sectioned rats which had larger testes than the others also had larger seminal vesicles, ranging from 75 to 380 mgs., while the weights of the other 12 were less than 60 mgs. each.

Histological sections of the testes of these stalk-sectioned rats for the most part revealed extreme atrophy with complete loss of spermatogenic activity and a marked decrease in interstitial tissue (Fig. 3). The largest testes of the stalk-sectioned group displayed less atrophy than the others. The least atrophic of the large ones had normal appearing interstitial tissue and showed active spermatogenesis in some tubules, though very few sperm were present in the lumens.

In the case of the 12 stalk-sectioned rats killed 75 days after operation, the average testes weight was less than that of either control group of rats (Table 1). All testes showed advanced stages of atrophy histologically. It should be noted, however, that the testes of these rats were slightly larger than those of rats sacrificed 35 days after stalk section and which showed only atrophic changes. A similar difference was noted between the weights of the seminal vesicles of these two groups of stalk-sectioned rats. The

seminal vesicles of these animals, however, were larger than those of rats which had been castrated for 75 days.

B. *Thyroid and adrenal glands:* Although the effect of stalk section on the structure and function of the thyroid glands and adrenal cortices will be covered more completely in another communication (Barrnett and Greep, in preparation), it is pertinent to note here that these organs of stalk-sectioned rats were smaller than those of normal controls. This is especially true of the adrenal which weighed about 50% of the normal value (Table 1).

C. *Growth:* Stalk-sectioning had the effect of decreasing the weight gain of the operated rats as compared to normal controls over the same period of time. On the average, the operated animals gained only 11.9 gm. in 35 days and the unoperated controls 102.5 gm. in the same period of time (Table 1). The 4 stalk-sectioned rats previously mentioned which had larger testes than the remaining 12 rats of the series killed 35 days post-operatively gained more weight than the others. However, the largest of these rats weighed 198 gm. whereas the smallest of the conclusion controls weighed 245 gm. The growth of stalk-sectioned animals killed 75 days after operation was also retarded.

D. *Changes in the pituitary stalk and hypophysis after stalk section:* Examination of serial sections 100 μ through the pituitary region of the specimens injected with India ink revealed that there was no regeneration of the hypophyseal portal blood vessels in any instance (Figs. 1 and 2). Instead, the vessels of the stalk remained interrupted and scar tissue filled the gap overlying the incision in the basisphenoid bone between these vessels and the anterior lobe. In the dissected specimens there was no continuity of stalk and pituitary gland.

Invariably there were gross and microscopic changes in the pituitary gland as a consequence of stalk section. Grossly the gland was shrunken,

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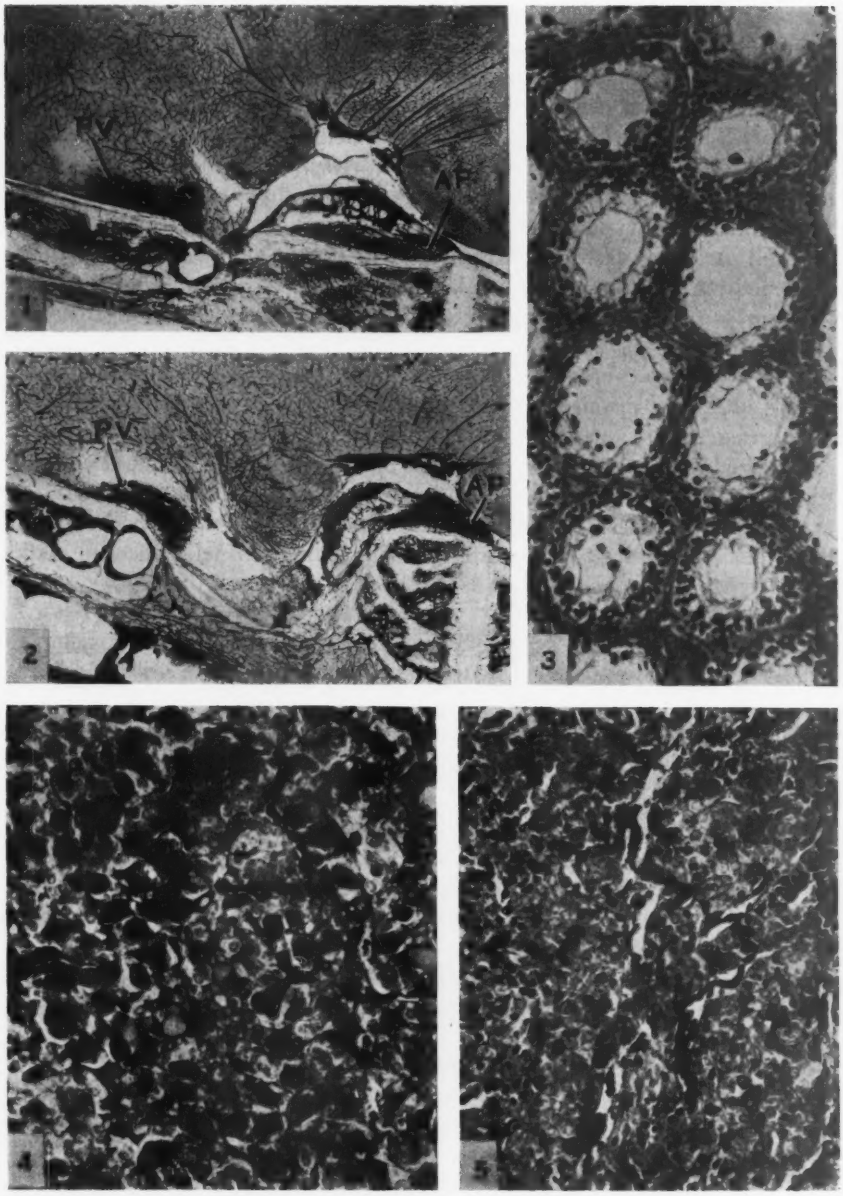
FIGS. 1 and 2. Median sagittal sections (100μ) through the region of the hypothalamus, stalk and pituitary gland. These sections were taken from two rats injected with India ink and are unstained. Note the well-injected sinusoidal loops and portal vessels (P.V.) in the region of the median eminence. There is complete lack of continuity between the blood vessels in that area and those in the anterior pituitary (A.P.). ×16

FIG. 3. Section of testis of stalk-sectioned rat stained with hematoxylin and eosin. Note the marked atrophy of the tubules. The cells persisting in their walls appear to be Sertoli cells. ×200

FIG. 4. Section of pituitary gland of castrated rat stained by the Heidenhain azan method. Large, well-characterized basophils are numerous. ×300

FIG. 5. Section of pituitary gland of stalk-sectioned, castrated rat stained by the Heidenhain azan method. There are only a few small cells suggestive of basophils present. ×300



FIGS. 1–5

friable and a dull red color in contrast to the pink of the normal gland. The glands always weighed less than those of unoperated control rats (Table 1). The salient histological features of pituitaries of stalk-sectioned rats were the replacement of cells in the central areas of the anterior lobe by connective tissue, and the widespread loss particularly of basophils but also of acidophils. The occasional chromophils which could be identified were located in the peripheral parts of the gland. Small chromophobes were the predominant cell type in the unscarred parts of the gland.

In all instances extensive atrophy of the neural lobe was noted and the intermediate lobe was usually enlarged and the cells basophilic.

II. *Gonadotropic potency of the pituitary after stalk section*

A. *Gonadotropic assay:* The injected suspension of ground pituitary glands of stalk-sectioned rats did not have enough gonadotropic potency to increase significantly the ovarian and uterine weights of the hypophysectomized recipient rats beyond those of the uninjected recipient controls (Table 2). The suspensions of pituitary glands from stalk-sectioned, castrated rats increased the weights of the ovaries and uteri of the recipients little, although more than pituitaries from animals which had been stalk-

TABLE 2. ASSAY DATA ON PITUITARIES OF CASTRATED, STALK-SECTIONED, AND STALK-SECTIONED, CASTRATED RATS

	Male donor rats			Recipient rats			
	No. rats	Average pituitary wt. (Mg.)	Average dose per rat acetone-dried pituitary (Mg.)	No. rats	Adrenals (Mg.)	Uteri (Mg.)	Ovaries (Mg.)
Castrated	9	13.8	0.68	9	10.3	86.7	55.4
Stalk-sectioned	8	4.7	0.60	8	10.8	15.1	9.7
Stalk-sectioned, castrated	16	5.3	0.75	16	10.7	26.0	12.1
Recipient controls				7	7.7	13.0	8.0

sectioned alone. In contrast, in those instances in which approximately half a pituitary taken from rats castrated for 75 days was injected, there was considerable increase in the weight of the ovaries and uteri of the recipients. Not only were the ovaries of these recipient rats very large but they had abundant corpora lutea indicating in support of the literature on castration that the pituitary of the castrate male rat stores large amounts of luteinizing as well as follicle-stimulating hormone. There was no signifi-

cant difference in weight of the adrenal gland of any of the injected recipient rats.

B. *Pituitary changes after castration:* The pituitary glands of rats which were castrated and stalk sectioned were larger than those of animals stalk-sectioned alone, but much smaller than those of rats which were only castrated (Table 1). The anterior pituitary glands of rats 75 days after castration were crowded with basophils, most of which were the typical large vacuolated "signet ring" cells, while others were smaller, having a more centrally placed nucleus and a negative Golgi image (Fig. 4). On the other hand, pituitary glands of rats which had been castrated and stalk sectioned had few basophils and there were virtually none in pituitaries of stalk-sectioned rats. The basophils of stalk-sectioned, castrated rats were small, round, rarely vacuolated and did not display a negative Golgi image (Fig. 5).

III. *Early changes in the pituitary glands after stalk section*

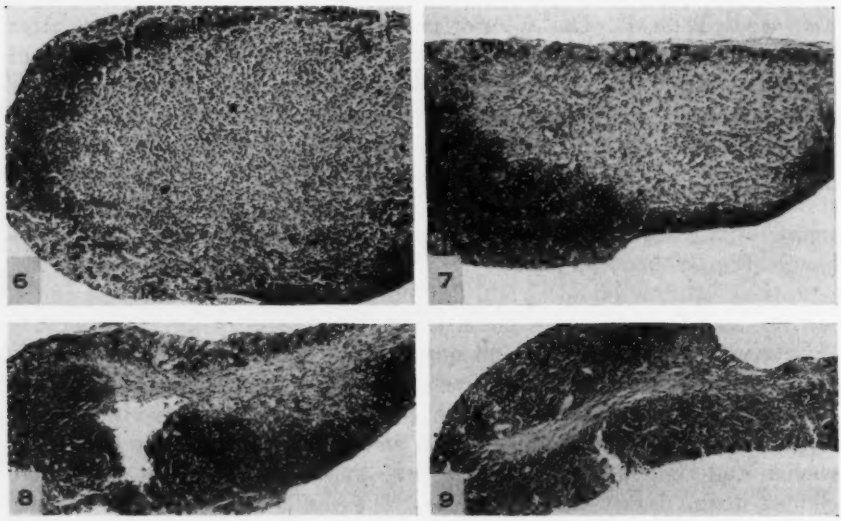
Extreme cellular necrosis and degeneration was noted in the anterior pituitaries examined 24 hours after stalk section (Fig. 6). In these specimens only a narrow peripheral rim of pituitary tissue appeared intact. No recognizable chromophobes were seen peripherally where some cells were swollen and stained poorly. Others were pyknotic, showing a condensed cytoplasm and darkly staining nucleus. The capillaries of the outer surface of the gland were engorged with blood cells, whereas no blood containing vessels were evident centrally.

Three days after stalk section the volume of peripheral swollen and pyknotic cells appeared expanded; yet the whole gland was diminished in size. No mitotic figures were seen in the peripheral regions. The central areas were relatively acellular (Fig. 7) containing scattered reticuloendothelial cells with engulfed material. In addition, nuclear remnants, a fibrous reticulum and an occasional swollen cell were also seen. The capillaries of the periphery appeared greater in number but no vessels were present as yet centrally.

Acidophils in small numbers and rare mitotic figures were seen for the first time in some stalk-sectioned pituitaries on the seventh day in the peripheral areas, scattered among the numerous small chromophobes. Further increase in the peripheral areas along with further overall shrinkage of the gland was apparent at this time (Fig. 8). Capillaries were seen at the edges of the relatively acellular central mass in greater abundance than heretofore observed. Increase in central connective tissue fibers was definite and scattered amongst the fibers were cells of probably reticuloendothelial and fibroblastic character.

The fifteen-day specimen was further shrunken, the central area was

compacted with connective tissue, and blood capillaries were now present centrally (Fig. 9). No further expansion of the outer cellular region was noted. The cellular area consisted mainly of small chromophobes scattered among which were occasional acidophils and still rare basophils.



The sections in Figures 6–9 were stained by the Heidenhain azan method and are magnified 85×.

FIG. 6. Section of anterior pituitary of rat killed 24 hours after stalk section. Note the widespread necrosis involving all but some peripheral portions of the gland.

FIG. 7. Section of anterior pituitary of rat killed 3 days after stalk section. The central area gives a washed-out appearance due to cellular necrosis.

FIG. 8. Section of anterior pituitary of a rat killed 7 days after stalk section. The gland has shrunk markedly and connective tissue fibers are evident in the central area. Such glands were especially friable.

FIG. 9. Section of anterior pituitary of rat killed 15 days after stalk section. The anterior lobe is now a shrunken image of its former self, and a moderately dense connective tissue scar is present in the central area.

DISCUSSION

The data presented indicate that gonad function was severely impaired in male rats as a consequence of section of the pituitary stalk. However, a few rats displayed histological evidence of a later incomplete return of testicular function after initial changes consisting of decrease in size of the testis and retraction of them into the abdomen. The testes of stalk-sectioned rats, although atrophic, nevertheless had a minimal degree of androgen activity, as shown in the seminal vesicles which were larger than

those of castrated rats but much smaller than those of untreated controls. Atrophy of the male gonads was more frequent and profound than that of the ovaries of the similar stalk-sectioned animals (Greep and Barrnett, 1951). Perhaps retraction of the testis into the abdomen after stalk section may have enhanced the incidence and extent of testicular atrophy as a result of increased temperature.

The influence of stalk section on the testis reported here support observations of Westman and Jacobsohn (1937, 1938, 1940) and Brolin (1945). Uotila (1939) on the other hand reported that the testes and seminal vesicles of stalk-sectioned rats were normal in weight and histology and showed no changes after exposure of the animals to cold. However, the testes of unoperated rats showed degenerative changes on exposure to cold. Furthermore, the testes and pituitary glands of operated and unoperated animals reacted alike to estrin injections (Uotila, 1940). Uotila discarded any stalk-sectioned rats which showed gonad atrophy or growth deficiency presumably because these animals had suffered extensive inadvertent damage to the anterior pituitary gland during sectioning of the stalk. In the light of our results, it seems possible that the animals which presumably suffered damage to the pituitary at the time of operation on the stalk were, rather, those in which successful stalk-sectioning was accomplished. In our instances there was no evidence of direct damage to the pituitary gland at the time of operation.

The gonad atrophy may be explained on the basis of the observed histological changes in the pituitary glands of the operated rats. Previous studies (Barrnett and Greep, 1951; Greep and Barrnett, 1951) and those reported here indicate that stalk section causes infarction of the anterior pituitary. Subsequent changes, such as shrinkage of the gland, capillary ingrowth from the periphery and production of scar tissue are consistent with the well-known sequellae of infarction of tissues. These results confirm observations by others who noted scarring and cellular changes of the anterior pituitary after stalk section (Crowe, Cushing and Homans, 1910; Mahoney and Sheehan, 1936; Rasmussen and Gardner, 1940; Westman, Jacobsohn and Hillarp, 1943; Brolin, 1945; and Harris, 1950) and after transplantation (Phelps *et al.*, 1939). The majority of the above authors, however, favored the view that the changes in pituitary function which they found were due to causes other than circulatory damage to that organ.

The possibility that a functional hypothalamic innervation is severed by sectioning the stalk has been emphasized by Westman, Jacobsohn and Hillarp (1943). Harris (1950) on the other hand interpreted gonad atrophy after stalk section as being due to interruption of the vascular pathway ordinarily traversed by an hypothalamic neurohumor which may control pituitary gonadotropic activity.

However, functional innervation of the adenohypophysis is not widely accepted (Rasmussen, 1938; Harris, 1948). The hypothalamic neurohumoral theory, although attractive, likewise does not seem to give a satisfactory explanation of our results. Our stalk-sectioned rats (Barrnett and Greep, 1950) had hypoactive adrenal and thyroid glands besides gonads, and showed deficiencies in growth suggesting total pituitary hypofunction. Furthermore, a partial return of gonad function was noted in a few male rats reported here, as well as in some female rats after stalk section (Greep and Barrnett, 1951) despite lack of regeneration of stalk vessels in these animals. Lastly, rats which were stalk-sectioned and castrated showed a small but nevertheless definite increase in pituitary gonadotropic potency over rats which were only stalk-sectioned. This would appear to indicate that the known pituitary gonadotropic relations are altered only quantitatively by stalk section, and that some release of these gonadotropins can occur in spite of both vascular and nervous isolation of the pituitary from diencephalic centers. We have also accumulated similar evidence related to the thyroid and adrenal glands of stalk-sectioned rats which reacted very sluggishly to a cold stress but nevertheless in a positive fashion (Barrnett and Greep, 1950). The degree of function which these infarcted pituitaries display may be dependent on the rate and degree of revascularization. Initial observations seem to indicate that this revascularization, in absence of regeneration of the portal blood vessels, occurs from the periphery of the gland and its source may be the blood vessels from the surrounding capsule of the pituitary.

In many respects, the panhypopituitarism caused by stalk section resembles that seen in some rats in which the pituitary gland was transplanted to other regions of the body. Hohlweg and Junkmann (1932) transplanted pituitary tissue into the kidneys of castrated rats and observed that "castration cells" did not appear in the transplant. They also observed that castration cells disappeared from castrate pituitaries transplanted into the kidneys of the castrated host. Because of these results, they postulated a sexual center in the hypothalamus which stimulated the production of gonadotropic hormones. Desclin and Gregoire (1936), on the other hand, emphasized certain changes in the basophils of pituitaries transplanted into the kidneys of castrated rats which they interpreted as a possible reaction to castration or to the unphysiological environment of the transplant. Furthermore, they demonstrated cytological changes in grafts after massive estrogen treatment. They concluded that pituitary grafts were not sensitive to small changes in circulating estrogens but marked elevations in estrogen titer would produce noticeable effects. In view of this work, Hohlweg (1936) modified his theory and agreed that sex hor-

mones could depress gonadotropic function by direct action on the anterior pituitary. Martins (1936) and Phelps *et al.* (1939) came to similar conclusions as their studies on transplanted pituitary glands indicated that estrogen acted directly on the anterior lobe and not through the nervous system.

SUMMARY

The effect of stalk section on the morphology and activity of the pituitary gland was investigated. Stalk section was found to cause anemic infarction of the anterior lobe, due to interruption of the circulation reaching it from the stalk, with subsequent shrinking of the gland, fibrosis and replacement of chromophils by chromophobes. The function of the damaged anterior lobe was impaired, as evidenced by atrophy of the testes, seminal vesicles, adrenals and thyroid glands and by decreased growth. Nevertheless, some of the stalk-sectioned rats displayed some degree of pituitary activity.

Experiments utilizing stalk-sectioned, castrated rats indicated that the changes in pituitary basophils and pituitary gonadotropic potency caused by castration were severely affected by stalk section but not completely prevented. These results demonstrated that the relationships between the pituitary gland and the gonads of male rats were altered only quantitatively, in spite of complete interruption of the nervous and vascular connections between the hypothalamus and the anterior lobe.

Acknowledgment

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IN VITRO CONTRACTURE OF PSEUDOPREGNANT UTERINE MUSCLE CONTRASTED WITH ESTROUS MOTILITY

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KEYE (1923) in Corner's laboratory observed the behavior of isolated strips of sow's uterine muscle at different stages of the cycle suspended in physiological saline solution. His graphs show that during the estrous phase, immediately before ovulation, the motility pattern is characterized by uniform contractions of high amplitude, occurring at intervals of 1 to 2 per minute. Almost at once after rupture of the follicles the spontaneous contractions begin to diminish in amplitude and to occur more frequently. As the organization of the corpus luteum progresses, the motility pattern changes from day to day until day 12 or 13 of the sow's cycle, when the embryos complete their journey through the reproductive canal to their sites of implantation and begin to attach themselves. At this time the motility pattern of the isolated muscle strip is characterized by very frequent spontaneous contractions (4 to 8 per minute) of very slight amplitude, so that the kymographic tracing becomes almost level.

Knaus (1927–1934) confirmed and extended these observations by studying the behavior of the excised uterine horns of the rabbit. Inasmuch as rabbits ovulate only after mating, in the normal course of events the effects of the corpus luteum can be seen only in pregnancy; but this difficulty can be obviated by mating the doe to a vasectomized male, thus inducing corpus luteum formation without impregnation.

The ensuing state of pseudopregnancy lasts for about 16 days, and resembles the corpus-luteum phase of the cycle in animals which ovulate spontaneously. Knaus observed that the excised uterine horn of the estrous rabbit exhibits a pattern of large, relatively infrequent contractions similar to those seen by Keye in strips of sow's uterine muscle. Thirty-two hours after a sterile mating, changes in the behavior of the uterine muscle, attributable to action of the hormone of the developing corpus luteum, can already be observed, and after a few days more the corpus-luteum phase pattern is fully established.

To facilitate similar observations during pregnancy Knaus ingeniously excluded the non-hormonal factors affecting the uterine muscle (distention by the fetuses, presence of the placentas) by cutting one oviduct, before

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mating the doe to a fertile male, so that one horn of the uterus remains empty. Such a sterile horn exhibits the same type of motility as in pseudopregnancy, except that the corpus luteum and the effects it produces upon the myometrium persist until the latter part of pregnancy.

Knaus moreover reported (1927, 1934) that when the uterine muscle of the rabbit is under the influence of the corpus luteum hormone it becomes insensitive to pituitrin, whereas in the estrous phase it responds by immediate contraction followed by a period of frequent contractions with incomplete relaxations as shown by a tetanus-like kymographic tracing. The refractory state produced by the activity of the corpus luteum is observable 48 hours after coitus (about 30 hours after ovulation) and lasts until the 16th day of pseudopregnancy or until late in gestation.

By using crude extracts of the corpus luteum Knaus (1930) produced in castrated female rabbits all the effects he had described in does with active corpora lutea. Makepeace, Corner and Allen (1936) completed the chain of evidence proving that progesterone is the active agent in establishing the progestational type of *in vitro* uterine motility and response to pituitrin, by producing these conditions in castrated does which they injected with chemically pure, crystalline progesterone.

Knaus considered the condition during the corpus-luteum phase to be one of low activity or inhibition. The uterus at 32 hours after coitus, he says "if isolated by the method of Magnus and Kehrer . . . shows a greater degree of flaccidity and a loss of power to contract spontaneously. It no longer responds normally to extracts of the posterior lobe of the pituitary body . . . at 48 hours after coitus it is very flaccid . . . From the tenth day onward its spontaneous movements quicken up again and become more powerful. The muscle, however, remains insensitive to pituitrin until the end of the sixteenth day of the pseudopregnancy . . . The corpus luteum, by neutralizing the effect of the hormone secreted by the posterior lobe of the pituitary body, brings about a marked slackening and sluggishness of the uterine muscle."

The above-mentioned observations and several others (see for literature Reynolds, 1949) suggest that the myometrium under the dominance of progesterone is different from that of the animal in estrus. The nature of the difference in behavior remains however quite unknown, and moreover it presents itself in various aspects under different experimental conditions, including both *in vitro* and *in vivo* techniques. In resuming the study of this important subject the present authors are primarily interested in the opportunity for biochemical analysis of myometrial contractility that is presented by the two contrasting motility patterns. Hence we have limited ourselves to the *in vitro* techniques used by Keye and Knaus, which we have merely improved and extended, in order to observe the contrasting

states under controlled conditions. For this reason no detailed reference will be made to the literature on *in vivo* experiments, for which the reader is referred to the comprehensive review of Reynolds (1949) and to Henry and Browne's summary of work on the effects of progesterone on the human uterus (1943).

As an introduction to the present studies it must be said that at present very little is known about the constituents of the myometrial cell or about the chemistry and energetics of the contraction-relaxation cycle. It is clear however from the work of one of us (Csapo, 1948; 1950 a, b), if one accepts the results and views of skeletal muscle biochemists, that the fibrous protein complex, actomyosin, is the contractile substance (in a final sense) in uterine, as it is in skeletal muscle. It has also been demonstrated that a quantitative interrelation exists between estrogen and actomyosin. With the help of iodoacetic acid poisoning and anoxia (and using the evidence of the Lohmann reaction) indications have been found (Csapo and Gergely, 1951) that in agreement with experiments on skeletal muscle, the immediate source of energy for myometrial contractility is derived from the nucleotide, adenosine triphosphate. Using the techniques described in the papers just cited, it has been found however that the characteristic cyclic differences in behavior of the myometrium do not depend upon differences in concentration of the actomyosin. The figures, in milligrams per gram of uterus, are as follows:

Sow:	
Estrus	Av. 4.3
Diestrus (corpus-luteum phase)	Av. 4.2
Rabbit:	
Estrus	Av. 6.2 (Csapo, in press)
Early pregnancy	Av. 7.6 (Csapo, in press)

METHODS AND MATERIAL

A modification of the Magnus-Kehrer method was used which was designed to permit observation and recording of spontaneous contractions as well as of response to electrical stimulation.

The physiological saline solution in which the uterine strips are suspended is contained in a Pyrex glass tube of 50 ml. capacity, closed at the lower end by a rubber stopper through which pass (1) a narrow-bore Pyrex tube serving as oxygen inlet, and (2) an electrode consisting of a similar Pyrex tube of narrow bore into which a platinum-iridium wire is sealed. This wire is formed into a hook at its free end, on which the lower end of the muscle is impaled. The upper end of the muscle is similarly transfixed by a hooked platinum-iridium wire connected to the writing lever. The lower hook is directly connected to one terminal of a low-impedence electrical transformer which delivers 60-cycle current, and the upper hook is connected to the other terminal by a highly flexible wire. The E.M.F. applied has been 20 volts. The stimulator circuit is closed by an automatic timer for 1 to 5 seconds at intervals of $\frac{1}{2}$, 1 or 2 minutes as desired, or at any time by manual operation.

The physiological saline solution is Burn and Dale's (1922) modification of Tyrode's fluid, as follows:

Distilled water	100 ml.
NaCl	0.90 gram
KCl	0.042
$MgCl_2$	0.0005
$CaCl_2$	0.024
$NaHCO_3$	0.05
Dextrose	0.05

The same fluid was used in Knaus's experiments. Its original pH is 7.50. After pre-oxygenation for 10 minutes to an oxygen concentration of 1.9 volumes per cent, the pH rises to 8.5. After the muscle is immersed in the bath the pH may rise in the course of several hours to 8.8. The experimental observations are made therefore between pH 8.5 and 8.8.

In order to exclude the possibility that the effects observed in these experiments were due to the relatively alkaline pH, in control observations the pH was lowered to 7.0–7.5. No change in muscle behavior was observed. In the case of the reported experiment (Fig. 3) the pH was lowered to 7.5 by using 37 mg. NaH_2PO_4 per 50 ml. bath.

In order to obtain samples of uterine muscle as nearly normal in physiological condition as possible, the rabbits were anesthetized intravenously with Nembutal, 40 mg. per kg. body weight. Ten minutes after the injection of the anesthetic the uterine horns were rapidly removed through an abdominal incision and placed in pre-oxygenated Burn-Dale solution of pH 8.5 at 20° C. This temperature inhibits spontaneous contractions and thus favors the cutting of rather uniform strips or segments from the uterine horn. In the entire set of experiments, strips 2.5 cms. long by about 0.5 cms. wide, cut longitudinally, were used; or if the uterus was small, whole segments of the horn of the same length were attached to the platinum hooks. Four strips were simultaneously set up in about four minutes from the moment of the abdominal incision. Even this relatively short period of time resulted in partial anoxia in the tissue, the significance of which will be discussed below.

The angle and length of the levers and also the load against which the strips were working have been standardized (lever horizontal when muscle fully relaxed; fulcrum to attachment of thread 4.5 cms., fulcrum to writing point 17 cms.; load 3 grams). All the graphs were reduced 30% in linear dimensions for publication.

Rabbits' uteri used in these experiments were obtained from mature virgin female animals weighing 3500 to 4500 grams. For the study of uteri in pseudopregnancy 10 does from the same stock were each mated to a vasectomized buck and were used 2 to 7 days after mating, within the time therefore when progesterone action on the myometrium can clearly be observed. Four segments from each rabbit were studied simultaneously and only those results will be reported here which were confirmed by comparable behavior of each strip of a given experimental animal. More than 50 rabbits were used in different reproductive conditions, mostly in estrus. The term "estrous rabbit" as used in this paper signifies a doe which was successfully mated immediately before the experiment. Acceptance of coitus by the doe indicates that the ovaries contain follicles available for ovulation and that consequently the uterus is in a state similar to that seen in other mammals at the time of estrus.

Conclusions derived from more than 50 experiments on uteri of the sow are reported because in the first place we were led to the observations and views presented here through work on that species, and secondly because the observations on the sow are

essentially in agreement with those on the rabbit and therefore have considerable corroborative value. The authors are indebted to the Schluderberg-Kurdle Packing Company of Baltimore for the material. Specimens were obtained at the abattoir from various stages of the cycle, i.e. estrus and the corpus-luteum phase. Using data published by Corner (1921), the dating with respect to the cycle was ascertained by the gross appearance and microscopical examination of the ovaries (follicles, corpora lutea) and of the endometrium. Strips of the circular muscle were studied with the same technique that was used with rabbits' uteri, but only two identical strips were used simultaneously.

Because of delays in the processing at the abattoir and time consumed in transportation to the laboratory, the uteri are unavoidably kept for many minutes under highly unphysiological conditions, in anoxia at less than body temperature. This interferes with their metabolism, resulting in a considerable loss of their energy stores. Yet we have been able by refrigeration at 4° C. to keep them in active condition for 7 days (since the uterus is able to rebuild the energy stores at least partially), and the contractile pattern was only slightly changed from day to day.

Any specimen, however, which has been out of control for the first few minutes after the death of the animal can be used in experiments only if the depletion of the energy stores is considered unimportant for the subsequent experiment.

In experiments on rabbits' uteri, in which the rapid techniques described above can be used, the period of readjustment is only 1 to 3 minutes. Since the behavior of the muscle-strips immediately after readjustment is critical for the present discussion, conclusions concerning this phase are drawn only from the rabbit experiments in which the specimens were transferred directly from the living animal to an oxygenated saline solution containing dextrose and the experiment was begun in 4 minutes.

OBSERVATIONS

Briefly summarizing the relevant part of our studies on estrous uteri, our observations on both rabbit and sow are in full agreement with those of Keye and Knaus. Spontaneous motility at estrus is characterized by relatively infrequent contractions of large amplitude, followed by prompt, complete relaxation. To this observation we can now add that under repeated electrical stimulus (5 seconds each minute) typical estrus-like spontaneous contractility can be imitated. The pattern obtained is more uniform, but the rate and amplitude are very similar to those occurring spontaneously.

We turn now to the corpus-luteum phase of the cycle. Because of the precautions we have taken as described above to place the uterine muscle in a favorable environment as quickly as possible, we have been able to observe significant behavior at the first stage of the experiment which might have otherwise escaped attention. As will be seen, the use of electrical stimulation has been a valuable aid in furthering our insight into the state of the muscle. An important accessory feature is the supply of enough oxygen to maintain a relatively high concentration in the bath (about 2 volumes per cent).

In order to reach optimal environment for *in vitro* studies of uterine strips a certain time must elapse between the beginning of the experiment

and the full activity of the muscle. In the case of abattoir material this time is about 15 to 30 minutes or even more and with the techniques described above for rabbits it is 1 to 3 minutes.

As mentioned above, when the strip or segment of rabbit's uterus is placed in the bath, a period of 1 to 3 minutes is required for adjustment During this time the optimal experimental temperature (37.5° C.) and an adequate oxygen concentration (about 2 volumes per cent) are reached

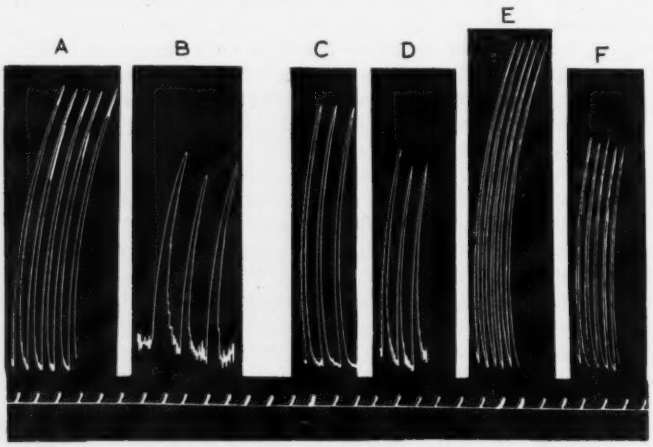

FIG. 1. Similarity of response of uterine strips to electrical stimulation in two species and in contrasting endocrine phases. A, sow in estrus, about day 27 of the 28-day cycle. B, sow in diestrus, about day 10. C, E, rabbit in estrus (10 minutes after mating). D, rabbit, in pseudopregnancy, 14 days after ovulation. F, rabbit in pseudopregnancy, 5 days after ovulation.

Electrical stimulation every half-minute (E, F), every minute (A, C, D), or every 2 minutes (B). In all experiments reported in this paper duration of stimulus was 5 seconds. All graphs reduced 30% from original size; the marks indicate minutes.

within the tissue, and the energy stores, partly exhausted during the brief period of at least partial anoxia, begin to be restored.

Immediately after a strip from an estrous uterus is placed in the bath, elongation of the muscle occurs and consequently the line of the kymograph tracing curves downward, leveling off to form a base line with which subsequent activity may be compared. Soon spontaneous contractions begin with increasing amplitude and thereafter continue for several hours, a contraction followed by prompt relaxation occurring at intervals of 1 to 2 minutes. If the muscle is stimulated electrically (20 volts, each minute for 5 seconds) after the spontaneous contractions have attained maximal amplitude, the stimulated contraction shows only a slight further increase of amplitude.

Uterine strips under the influence of progesterone, on the contrary, do not elongate at the beginning of the experiment, but instead undergo slow shortening. The unavoidable period of anoxia before the start of the experiment evidently affects the estrous and the progestational muscle in

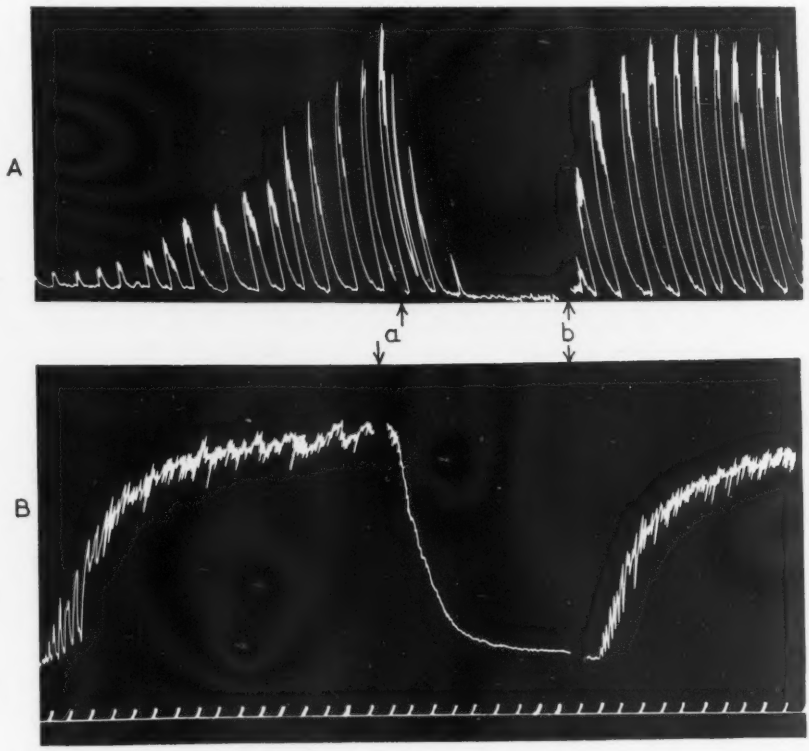

FIG. 2. Gradual appearance, at beginning of experiment, of spontaneous contractions of sow uterine strips in estrus, about day 27 of cycle, A; and of spontaneous "contracture" in diestrus, about day 10 of cycle, B. Rapid abolition of spontaneous contractions and contracture respectively in anoxia (*a*) and rapid return of both in oxygen (*b*).

different ways. The spontaneous contractions moreover begin almost immediately with a rapid rate of 3 to 8 per minute but they are very small in amplitude. Knowing that the contractile "skeleton," the actomyosin-ATP system, is not significantly different in the two types of muscle, one would expect the progestational muscle to have the power of contraction to full amplitude. Electrical stimulation justifies this expectation, confirming the conclusions drawn from chemical determinations. As illustrated in Figure 1,

sows' uteri in the corpus-luteum phase of the cycle and rabbits' uteri in pseudopregnancy respond to electrical stimulation by full contractions resembling those elicited from estrous uteri. The differences in behavior,

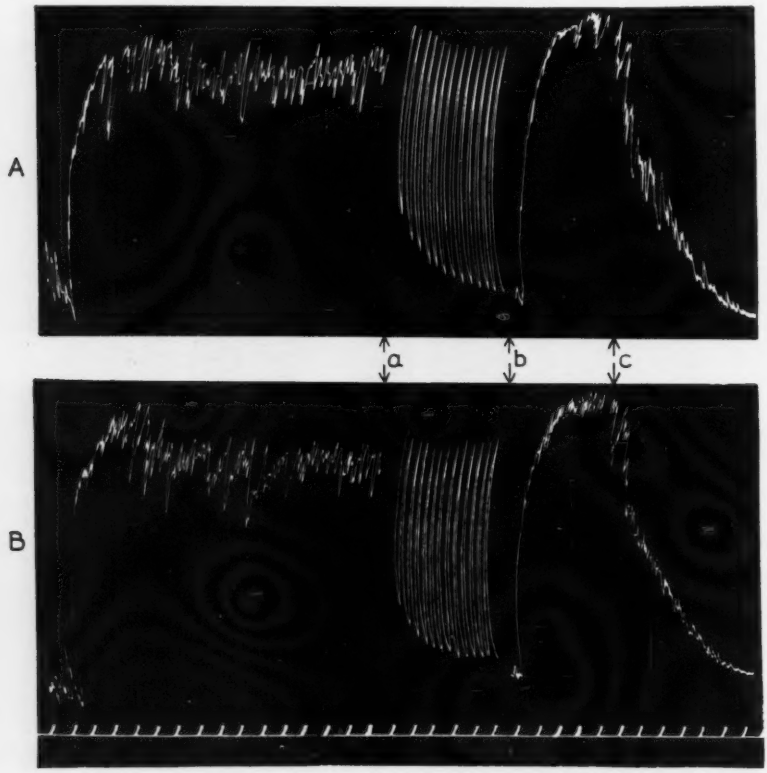

FIG. 3. Spontaneous contracture of rabbit uterine strips in pseudopregnancy. A, pH of bath 8.5; B, pH 7.5. After a long period of full contracture, electrical stimulation at half-minute intervals is begun, with immediate suspension of the contracture (*a*). After electrical stimulation is discontinued the spontaneous contracture returns (*b*). At the peak of contracture anoxia is instituted (*c*), resulting in suspension of contracture.

therefore do not result from any basic difference in the actomyosin-ATP system.

If the muscle is allowed to contract spontaneously, about 3 to 30 minutes (depending on the condition of the muscle before the experiment) from the beginning of the experiment a sharp change in the pattern of contractility sets in. This would be quite unexpected on the assumption that spontane-

ous contractility of the myometrium is inhibited by progesterone. What actually happens (Fig. 2, B) is that at each successive contraction the muscle shortens more and more but relaxes less and less. When no further

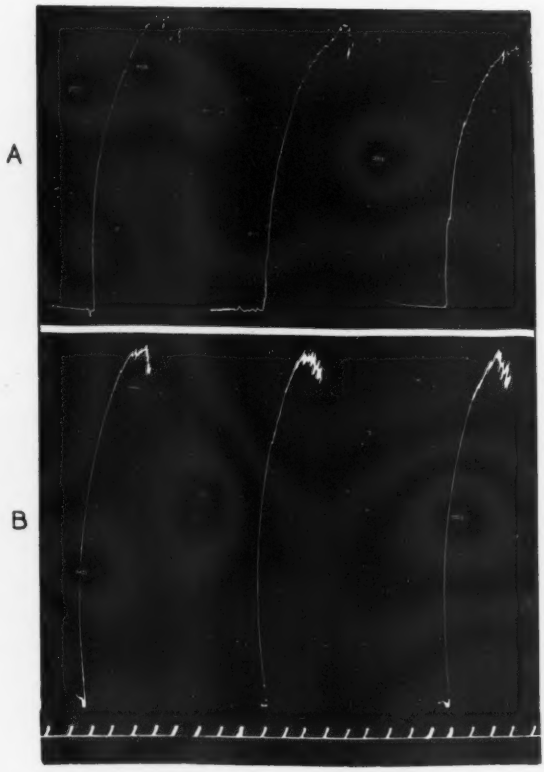

FIG. 4. Relation between oxygen saturation and the speed of development of spontaneous contracture. When oxygen saturation is 1.9% by volume (B), the contracture develops immediately after suspension of the previous contracture by a single (unrecorded) electrical shock. Spontaneous contracture develops about four times more slowly if the oxygen flow is four times reduced (A). Experiment repeated 3 times.

shortening is possible, the tracing reaches a high plateau on which frequent waves of small contractions and relaxations are superimposed. The condition thus reached will be called "contracture," an extension of terminological usage which we think is justifiable. It must be noted that the contracture may be interrupted temporarily by definite though incomplete periods of relaxation. Such interruptions of the state of contracture oc-

curred irregularly in our experiments, and not in every case. We are therefore not able at present to explain them.

If at the peak of contracture anoxia is produced (by saturating the bath

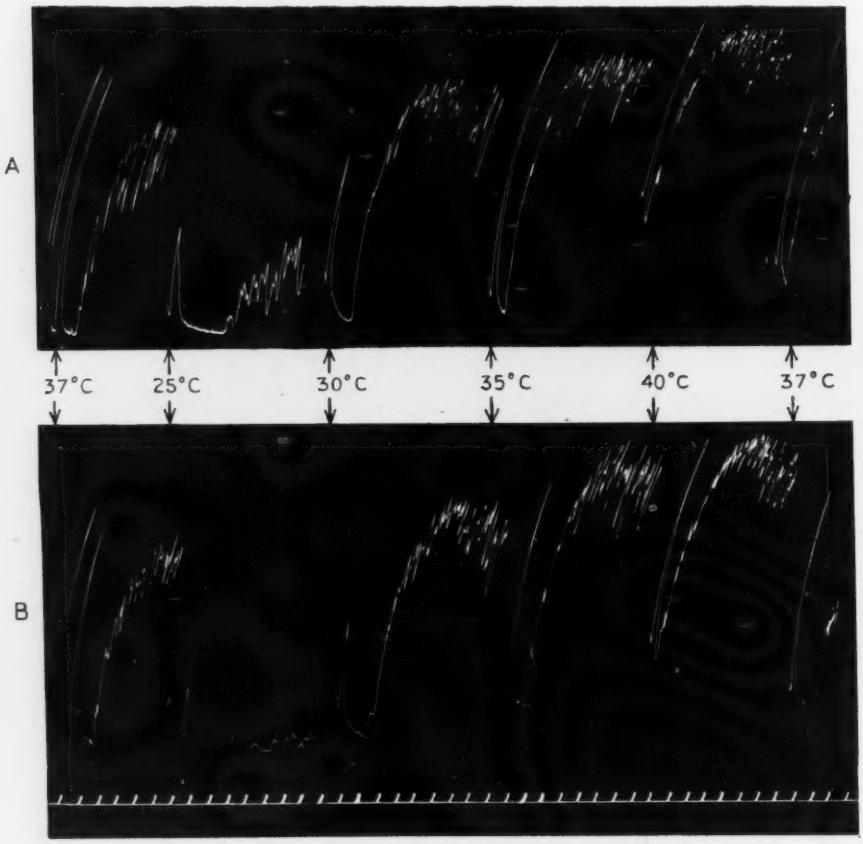

FIG. 5. Relation between temperature and the speed of development of spontaneous contracture. Identical strips (A and B) from a 6-day pseudopregnant rabbit are used. (The highly similar behavior of the third and fourth strips used in these experiments is omitted from this and other graphs.) One or two contractions are produced by electrical shocks at the beginning of each run, after which spontaneous contracture develops at different rates closely depending upon the temperature.

with nitrogen), a suspension of the contracture occurs and the tracing returns to the base (Fig. 2, B). If the bath is again saturated with oxygen, contracture returns.

For a closer inspection of this phenomenon we must turn particularly to

the rabbit experiments. As indicated in the introductory part of this paper the progesterone-dominated uterus can most easily be obtained and studied in pseudopregnant rabbits.

Figure 3 illustrates several characteristic features of the progesterone contracture which will be discussed in the following paragraphs. Chief among these is the remarkable fact that the contracture is at once released by electrical stimulation (Fig. 3, *a*) which causes the muscle to relax completely. After cessation of the stimulus, contracture again sets in.

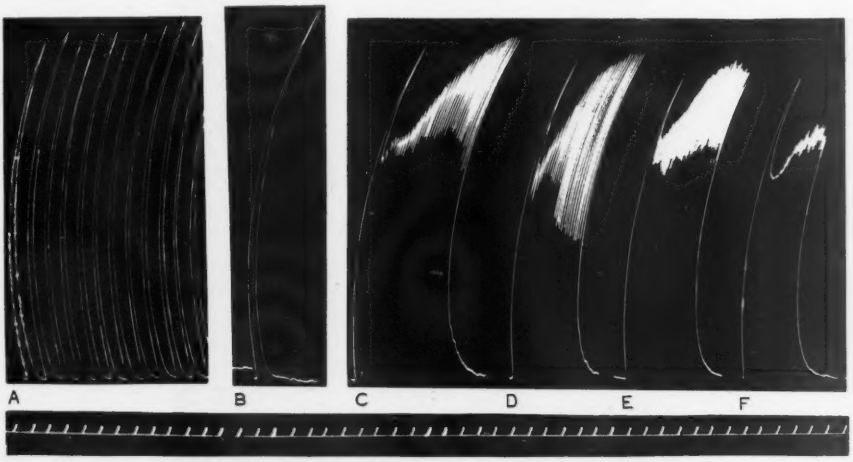

FIG. 6. Imitation of "progesterone contracture" of estrous uterine strip by the use of frequent electrical stimulation. A, spontaneous contractions, about 1 per minute. B, a single electrical shock elicits a response similar to the individual spontaneous contractions. C, electrical stimulation repeated at a frequency of 6 5-second periods per minute. D, the same. E, frequency increased to 15 5-second periods per minute. F, continuous stimulation for four minutes. Suspension of the contracture immediately follows the cessation of electrical stimulation.

This figure shows also, by comparison of two strips working in baths at pH 7.5 and pH 8.5 respectively, that the contracture, like other features of the motility pattern, does not depend upon the relatively alkaline state of the Burn-Dale saline solution.

That the effect is not accidental is proved by its ready repetition in successive animals from the 46th hour to the 7th day of pseudopregnancy (as far as our experiments go at present); by its similarity in as many as four strips from the same animal studied simultaneously; and by the repeated use of the same strip, suspending contracture temporarily by a single electrical shock or with the help of anoxia.

It has also been demonstrated (Fig. 4) that oxygen saturation has a direct relationship to the rapidity of development of this contracture. Temperature also has a direct relationship to the speed of the development of this contracture (Fig. 5). Contracture develops very slowly and incompletely at 25° C. At 40° C. the contracture begins immediately after the response to a single electrical shock is completed. (Electrical shocks are conveniently used to suspend previous contracture, to indicate the base

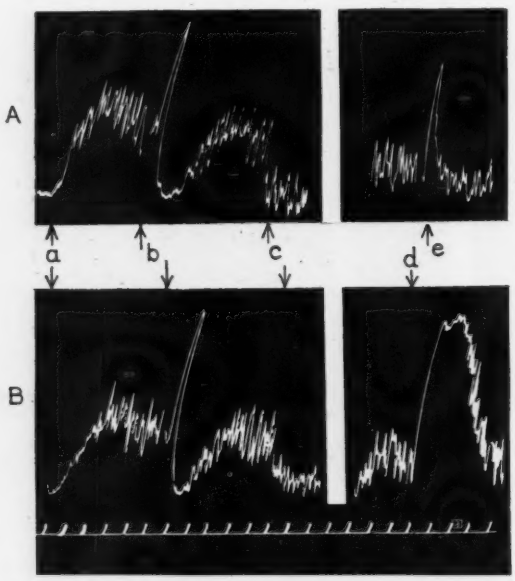

FIG. 7. Incomplete contracture of 46-hour pseudopregnant rabbit (A, B). Incomplete contracture develops spontaneously (*a*). Contracture suspended by a single electrical shock (*b*), after which spontaneous contracture again develops. One gram extra load results in a return to the base line (*c*); space indicates the release of the extra load, which results in the return of incomplete contracture. At *d*, 0.1 ml. 1/10,000 adrenaline added to bath with positive effect. At *e*, one unit Pitocin added to bath with positive effect.

line, i.e. the potential degree of relaxation when not in contracture; and to mark an instant from which the onset of spontaneous contracture may be timed.) At this temperature, however, the base line rises and the amplitude decreases. Probably slight but irreversible changes take place in the tissue, as suggested by the last record after the temperature is again decreased to 37° C.

All these observations indicate that the *in vitro* "progesterone contracture" is an active, reversible process, which needs oxygen for its develop-

ment and is suspended by anoxia or temporarily by an electrical shock. Experiments also suggest that this contracture is the result of a chemical process or a series of processes, the rate of which is increased by increasing the temperature and oxygen concentration.

It is also clear that in this condition the spontaneous contractility is not inhibited. On the contrary it appears that the contracture is a kind of

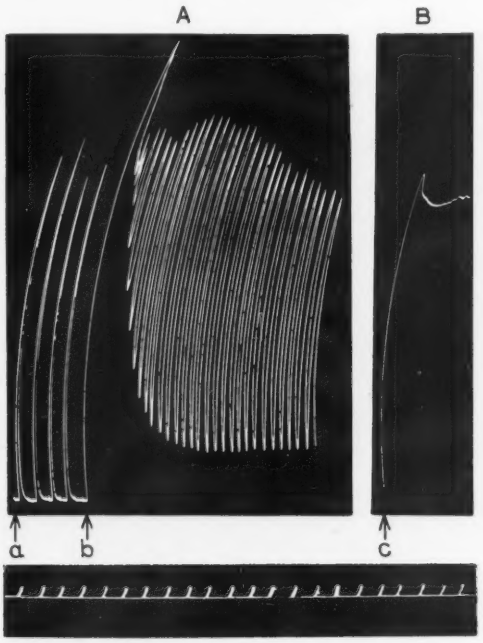

FIG. 8. Effects of Pitocin and adrenaline on estrous uterine strips compared. *a* = spontaneous contractions. At *b*, 1 unit Pitocin added to bath. At *c*, 0.1 ml. 1/10,000 adrenaline added to bath.

summation of successive spontaneous contractions, one following another before the intervening phase of relaxation is complete so that progressive shortening goes on to more or less persistent and complete contracture. The simplest hypothesis for the development of this contracture would be to suppose that it results from a high rate of spontaneous stimulation or from increased sensitivity of the muscle to the stimulus, whatever it is, that causes spontaneous contraction. The authors have succeeded in imposing contractures on estrous muscle by using a rapid succession of brief 60-cycle shocks (6 and 15 per minute) or continuous 60-cycle stimulation for several minutes (Fig. 6). In view however of the present lack of knowledge

of the spontaneous contraction mechanism, we are not convinced that the problem is as simple as this imitation of contracture might suggest.

There are intermediate stages between estrus and full progesterone domination. Such a condition was found in the uterine strips of a rabbit in the 46th hour of pseudopregnancy (Fig. 7) which exhibited incomplete contracture, i.e. if at the peak of contracture electrical stimulation was applied, the amplitude of the electrical response increased much further,

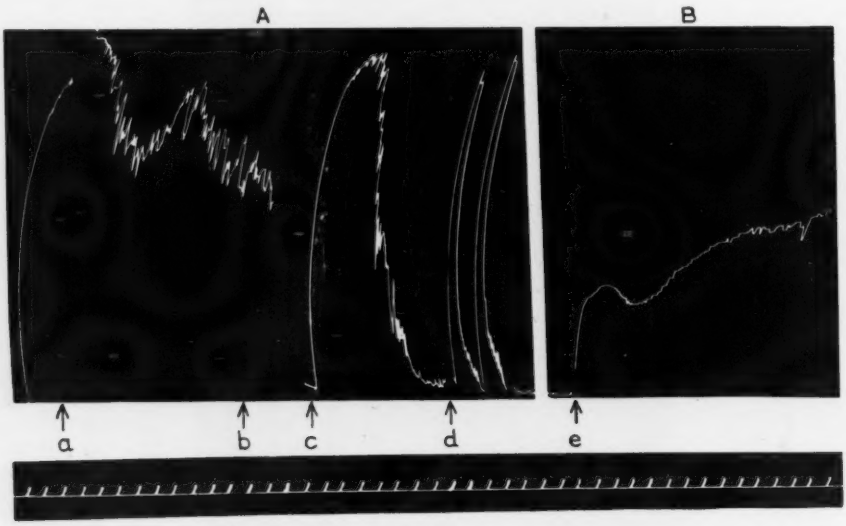

FIG. 9. Response of pseudopregnant uterine strips to adrenaline. *a*, effect when contracture is fully developed (mark indicates adrenaline administration). After change of the bath, one electrical shock (unrecorded) at *b* suspends contracture. Spontaneous contracture develops again (*c*) and becomes suspended spontaneously. Two electrical shocks (*d*) demonstrate full capacity of the strips at the end of the experiment. *B*, development of spontaneous contracture delayed by reduced oxygen flow ($\frac{1}{4}$ of the standard) after previous anoxia. Before spontaneous contracture develops, adrenaline is administered as before (*e*).

indicating the incompleteness of the previous spontaneous contracture. Such a contracture is very weak and can be reversed by 1 gram extra load. With a 3-day pseudo-pregnant uterus this contracture-suspending effect was obtained with 6 grams extra load. During this transition stage, adrenaline added to the bath induces a full, but temporary contracture as in the estrous uterus. Pitocin results in moderate contraction, which is, however, not followed by a contracture (with increasing relaxation) as in the case of the estrous uterus (cf. Fig. 8). All these findings suggest a kind of

intermediate state between estrus and full development of pseudopregnancy.

This type of response to adrenaline and Pitocin can be observed however only in early pseudopregnancy, when the contracture itself is incompletely developed. When the contracture is complete (Fig. 9) no such effect can be expected, since no further increase in amplitude (and therefore in the degree of contracture) is possible. If adrenaline is administered at the peak of contracture, no further increase being possible, the muscle remains

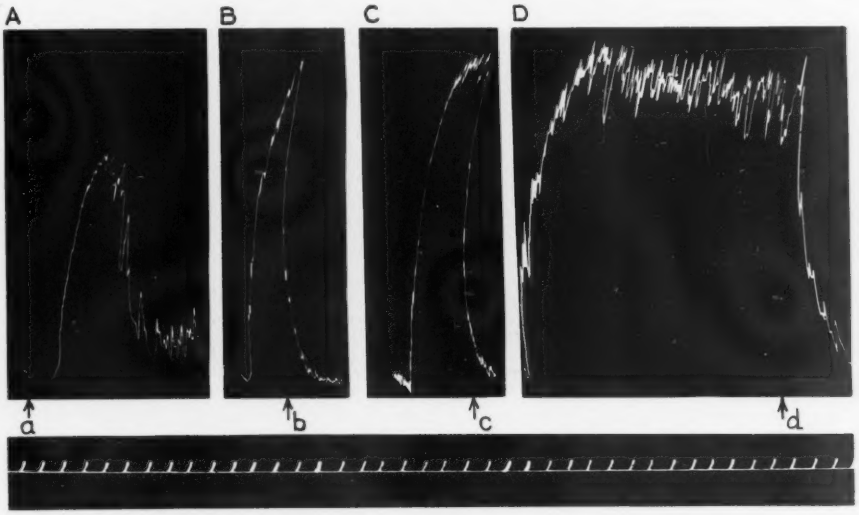

FIG. 10. Effect of Pitocin on pseudopregnant uterine strips. *A*, delay in the development of spontaneous contracture by reduction to $\frac{1}{4}$ of the standard oxygen flow. Before contracture develops 1 unit Pitocin per 50 ml. bath is administered (*a*). B, C, D: with standard oxygen flow Pitocin (as before) is administered at the peak (*b*), shortly after (*c*), and long after (*d*) contracture has been fully developed. Note immediate contracture-releasing effect.

in contracture as it would without adrenaline, so that the effect of the drug cannot be distinctly observed. If after change of the bath, contracture is then suspended by one electrical shock, contracture develops spontaneously but the tracing returns quickly to the base line. This release of the contracture must in some way be a result of previous adrenaline administration. If the drug is administered shortly after the suspension of previous contracture by anoxia (and if the muscle is given insufficient oxygen supply in order to delay the recurrence of contracture), spontaneous contracture occurs as if the drug had not been given, so that the result is again not conclusively an adrenaline effect.

As far as the effect of Pitocin is concerned, we are not in accord with Knaus and with those who support his view that the posterior pituitary oxytocic hormone does not affect the behavior of pseudo-pregnant uterine strips *in vitro*. We have observed a definite effect of Pitocin when administered in doses of 1 international unit in the 50 ml. bath. This effect however is in the opposite direction to that observed on the estrous uterus. If the drug is administered before the development of spontaneous contrac-

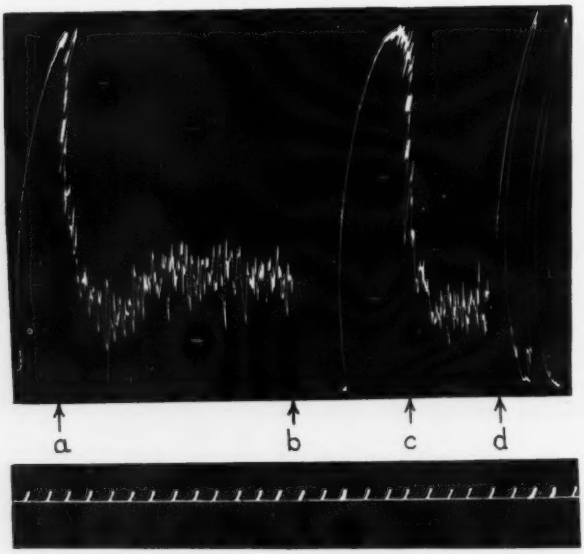

FIG. 11. Temporary spontaneous contracture in the presence of traces of Pitocin. After spontaneous contracture develops, 1 unit of Pitocin per 50 ml. bath is administered (*a*). Long after the contracture-suspending effect occurs, the bath is changed (*b*). Spontaneous contracture develops again, but holds only temporarily; then the Pitocin effect occurs without the further addition of Pitocin (*c*). Electrical shocks indicate the full capacity of the muscle at the end of the experiment (*d*).

tions, no effect can be observed for a time (Fig. 10, A). The spontaneous contraction develops as it would without the administration of the drug, and then unexpectedly a rapid return to the base line occurs instead of the continuance of contracture. If the drug is administered either immediately or after the contracture has existed for a time, the result is immediate suspension of the contracture (Fig. 10, B, C, D). This paradoxical effect will be called hereafter "Pitocin reversal." If the bath is changed and the Pitocin thus removed, traces of the drug may remain, changing the behavior of the muscle, so that spontaneous contracture develops fully but

only temporarily and thereafter spontaneous suspension occurs (Fig. 11).

The contracture-releasing effect of pituitrin was actually observed by Robson (1932), but because he did not realize that he was working with muscle in a state of contracture (as his graphs indicate) the effect was considered by him to be "relaxation." Makepeace, Corner and Allen (1936) also observed marked "relaxation" after the addition of pituitrin, but they also had overlooked the existence of contracture.

DISCUSSION

The observations we have reported differ from those quoted from previous workers on two major points. We have described the state of the uterine muscle under the domination of progesterone and under our experimental conditions as one of contracture rather than of relaxation; and we have found that in this state the muscle responds actively to the posterior pituitary oxytocic hormone by suspension of the contracture.

With respect first to the contracture, which invariably occurred in our experiments, the question arises, how is it possible that it was not previously recognized? The fact that previous workers have not published graphic records of the first parts of their experiments, when the muscle strips were settling down to a steady pattern of motility, indicates that attention was not given to this phase. If we disregard the early parts of our own records, taken while the contractures were developing, and if we do not indicate the base line, our graphs are similar to those of previous observers. It is usually not possible to ascertain from the records of other workers whether their experimental strips were actually in contracture or whether on the other hand the contracture did not develop at all in their experiments. The published records do not show the original base line before the spontaneous contraction began in a given strip, which would decide between the two possibilities by indicating the actual condition of the muscle during the observed part of the experiment. The use of anoxia or electrical stimulation, which would have cleared up the question by causing suspension of the contracture, did not occur to earlier experimenters.

Turning now to the discrepancy regarding the action of pituitrin, it is clear that if the drug is added to the bath when the muscle is in contracture, no further contraction can occur and in this sense the oxytocic substance will appear to be ineffective.

In the case of other studies it is possible that the muscle was receiving insufficient oxygen. Unfortunately the oxygen concentration of the physiological saline solution used by previous investigators was not reported. We found that if we reduce the oxygen concentration of about 1.9 volumes per cent (our standard) to one-fourth of that level, the development of

contracture is delayed (Fig. 10). The delay may amount to no more than 5 to 10 minutes if the strips are transferred directly from the living animal into the experimental bath in as short a time as four minutes. But in the case of muscle from the abattoir in which the muscle has been in complete anoxia for many minutes (e.g. abattoir material or rabbits' uteri subjected to experimental delay) the postponement of contracture may well exceed 30 minutes. In this highly unphysiological condition (relative anoxia, incomplete energy stores) the strip certainly can appear to be relaxed or inhibited. In this state the muscle does not respond to pituitrin, and is therefore not in condition to show either the usual effect or the reversal. Similar considerations when fully understood will probably explain also some of the discrepancies in the literature of *in vivo* observations on uterine motility, in which also anoxia and incomplete energy stores may be involved.

If we knew more about the basic reactions involved in the spontaneous rhythmicity and the contraction-relaxation cycle of the uterus, we should be able to locate more definitely the effect of progesterone and that of Pitocin. The fact that electrical stimulation produces contractions of progesterone-dominated muscle equal in amplitude to those of estrous muscle suggests that the contractile systems are similar in each. Consequently progesterone probably in some way affects the reactions responsible for spontaneous stimulation or alters the response to the stimuli. This hypothesis is based on experiments, mentioned above, which show that estrous muscle can be brought into similar contracture by frequent electrical stimulation (6 to 15 stimuli per minute); that anoxia (which inhibits spontaneous stimulation in estrous muscle) suspends the contracture of progesterone-dominated muscle without abolishing electrical response; and that a single electrical shock temporarily reverses this contracture, probably by producing a kind of refractory period which cannot be broken through by relatively ineffective spontaneous stimuli.

It has been reported (Csapo, in press) that the rate of spontaneous rhythmicity is dependent on temperature in the case of estrous muscle. Some unfinished experiments suggest that progesterone-dominated muscle at about 20° C. exhibits a slow rhythm of contraction similar to that of estrous muscle at 37° C. At this low temperature, therefore, the relaxation phase is more complete (estrus-like), but unfortunately the reduction of amplitude (due also to the low temperature) does not favor the demonstration of this fact. The observation now reported, that the rapidity of development of contracture is also dependent upon temperature as well as upon oxygen saturation, indicates that the contracture is the result of a chemical process which needs oxygen for its full development. The reversibility with anoxia, electrical stimulation and Pitocin indicates that irreversible

changes do not occur in the myometrial cell when contracture develops.

We believe that the foregoing observations, although made under carefully limited experimental conditions, are sufficient to suggest that contracture of the uterine muscle under the influence of the corpus luteum hormone reflects fundamental characteristics of the myometrial cell. Whether our concepts regarding the nature of the contracture will be useful in renewed study of uterine motility in the living animal and human subjects cannot now be surmised. For the present we are utilizing our findings in an intensive study of the mechanism of the contraction-relaxation cycle and the energetics of the myometrium in the two contrasting states of estrus and pseudopregnancy.

SUMMARY

1. Using excised uterine strips *in vitro*, it has been shown that uterine muscle of the sow in the corpus-luteum phase of the cycle and of the rabbit in pseudopregnancy respond to electrical stimulation in all respects similarly to that of the same species in estrus.

2. The uterine muscle of both species when under the dominance of progesterone spontaneously develops a state of contracture and contrary to previous reports is not relaxed or inhibited.

3. This contracture is reversible and can be suspended by electrical shock or anoxia.

4. The oxygen tension in the bath and the temperature have a direct relationship to the speed of development of the contracture.

5. Using frequent electrical stimulation (6 to 15 per minute) the progesterone contracture can be imitated with strips from estrous uteri.

6. Incomplete contracture has been observed on the second day of pseudopregnancy, representing an intermediate stage between estrus and the full development of pseudopregnancy.

7. Response to adrenaline cannot be observed distinctly if contracture is fully developed.

8. Pitocin has a definite effect on pseudopregnant uterine muscle, producing a suspension of the progesterone contracture. This action is the reverse of that exerted upon estrous muscle.

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ACCELERATED GROWTH OF THE THYROID IN NORMAL AND "HYPOPHYSECTOMIZED" FETAL RATS GIVEN THYROTROPHIN[1,2]

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THE observations to be presented show that the prenatal growth of the thyroid may be stimulated by introducing thyrotrophin into the fetal circulation. This effect may also be demonstrated in fetuses deprived of the hypophysis by removing that portion of the head which is above the tongue, lower jaw and foramen magnum in such manner that the thyroid and its blood vessels are spared.

MATERIALS AND METHODS

In rats of the Sprague-Dawley strain, 52 pregnancies were dated from the time of observed copulation. Four groups of fetuses were used. *Group A* consisted of two normal controls taken at 18½ days from one mother and two taken at 19½ days from a second mother. *Group B* consisted of 42 controls from 35 litters in which experiments had been performed. These controls had been left untouched in the uterus, and were obtained at autopsy, near term. *Group C* (*given thyrotrophin*) consisted of 79 fetuses, one or two from each of 39 litters. Treatment was begun on the morning of the 20th day, when the fetuses were about 19½ days old. Each fetus was removed from the uterus (but left attached to it by the umbilical cord), was given the first of a series of four 0.05 cc. subcutaneous injections of thyrotrophin and was then placed in the body cavity of the mother (method of Wells, 1950). The succeeding injections were made at intervals of about 12 hours. Two preparations were used, Ayerst's Factor 456 in a concentration of 50 U. per cc. and Armour's Lot 2Cr3 in concentrations of 2.0, 1.0 and 0.5 mg. per cc.[3] When the experiments were terminated, at about one hour before expected term and 52 hours after the first injection, it was found that 12 fetuses had survived (17%); this period of 52 hours is about one-tenth of the total period of gestation. Of these 12 fetuses, 5 had received T-Ayerst and 7 T-Armour (Table 1). *Group D* (*"hypophysectomized" and then given thyrotrophin*). This group consisted of 21 fetuses (one or two from each of 11 litters), and the treatment was like that in the preceding group in all respects except that prior to the first injection each fetus was deprived of the hypophysis by subtotal decapitation. In performing this decapitation, curved iridectomy scissors were used. The plane of section is best described as starting at the left angle of the mouth, extending dorsally to a mid-

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[2] This study was supported in part by grants: 1) from the National Institutes of Health, Public Health Service and 2) from the Medical Research Fund of the Graduate School.

[3] These preparations were supplied by Dr. J. Murray Scott of Ayerst, Mc Kenna and Harrison, Ltd. and by Dr. Edwin E. Hays of Armour and Company.

dorsal cutaneous point opposite the foramen magnum and ending ventrally at the right angle of the mouth. Thus the hypophysis and subjacent portion of the sphenoid bone were removed, while the blood vessels of the thyroid and most of the lower jaw were spared. During the ensuing hemorrhage, the clotting of the blood was so rapid that ligatures were not used. Each of the 21 "hypophysectomized" (subtotally decapitated) fetuses was then given a series of subcutaneous injections of thyrotrophin. At autopsy, near term, three of the 21 fetuses had survived (14%).

TABLE 1. VOLUME OF THE LEFT HALF OF THE THYROID

Group	Number of fetuses*	Treatment			Body weight, gms.	Volume, $mm.^3 \cdot 10^2$	Vol. $(mm.^3 \cdot 10^2)$ / Body wt. (gm.)	
		Thyrotrophin		Period, hrs.			Average†	P_t‡
		Kind	Total amount					
C	5E	T-Ayerst	10 U.	52	3.408	33.23	9.75 (7.89–12.69)	.005
	5C				4.013	14.64	3.65 (2.88– 4.62)	
	7E	T-Armour	.09–.40 mg.	49	3.716	20.09	5.41 (4.03– 6.31)	.003
	7C				4.236	15.87	3.75 (3.44– 4.15)	
D	2E	T-Armour	.08–.10 mg.	45	2.741§	12.40	4.52 (3.97– 5.43)	
	2C				4.109§	13.25	3.22 (2.80– 3.69)	

* E, experimental; C, litter-mate controls of Group B.
† Numbers in parentheses indicate the range.
‡ Results of the t-test; any value smaller than .01 would suggest that the several differences in a group (C minus E) are highly significant (Treloar, 1942).
§ Headless body weight.

At the conclusion of the experiments the fetuses and controls of the same sex were removed, weighed, exsanguinated, prepared for fixation (by exposing the thyroid) and placed in Bouin's solution. After fixation, the thyroid was viewed under a dissecting microscope and removed with sharpened jeweler's forceps.[4] The removed thyroid was vertically bisected by cutting through the middle of the isthmus. The left half of the gland was sectioned in series at 10μ, and the sections were stained with hematoxylin and eosin. The right half was sectioned at 3μ, and selected sections were stained with Weigert's iron hematoxylin without counterstain.[5]

The stained sections of the several thyroids were compared microscopically with the aid of a microcomparator. Then the volume of the left half of the thyroid was determined from the series of sections (every sixth section) by the paper-weight method (Hammar, 1914; Boyden, 1940).

OBSERVATIONS

Groups A and B. It was not possible to detect any morphological difference between the thyroids of male fetuses and those of female fetuses. At 18½ days the gland had a thin capsule, abundant parenchyma and a small amount of loose connective tissue (Group A). Most of the parenchyma was composed of solid cords of cells, but in places, especially in the peripheral portion of the gland, there were a few, small, luminated follicles. At 19½

[4] During this process, the thyroid of one fetus of Group D was accidentally lost.

[5] In preliminary work some of these 3-μ sections were stained by a modification of Masson's trichrome technique (Goldner, 1938), but this was abandoned because the cells and colloid were not stained with that sharpness of differentiation which was observed in control sections of the thyroid of an adult rat.

days the luminated follicles were somewhat larger and more numerous than before, but were still mostly restricted to the peripheral portion of the gland. All controls of Group B were alike in that the determined age was about 21 days and 15 hours (16 ± 1 hours). There were luminated follicles in all parts of the gland, peripherally (Fig. 2) and centrally as well. The follicular epithelium was low cuboidal, and the lumina contained a lightly stained, non-vacuolated substance (colloid). In body weight and volume

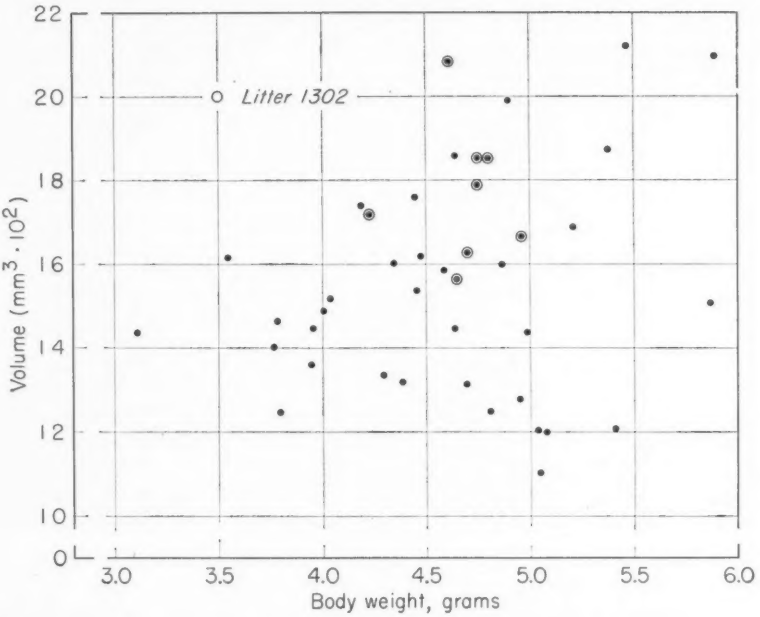

FIG. 1. Scattergraph of the volumetric data on the left half of the thyroid of the 42 controls of Group B. Eight of these fetuses were from one litter (circled dots).

of the thyroid, the range of variation in 8 fetuses from one litter was smaller than that in the other fetuses of Group B (one from each of 34 litters, Fig. 1).

Group C. The growth of the thyroid had been speeded up (Table 1). Sections of the gland showed that the parenchyma was "tightly packed," that the follicles were evenly distributed throughout the gland and that the follicular epithelium was higher than that of controls (Fig. 3). In an occasional follicle, like the one nearest the lower left corner of the photograph, the colloid in the lumen was vacuolated.

Group D. That each fetus had been successfully deprived of the hypophysis was determined by dissection (by exposing the hypophysis in that

part of the head which had been removed and preserved in formalin). One fetus had been given a total of 0.10 mg. of T-Armour in four injections, while the second fetus had been given only 0.08 mg. in three injections.

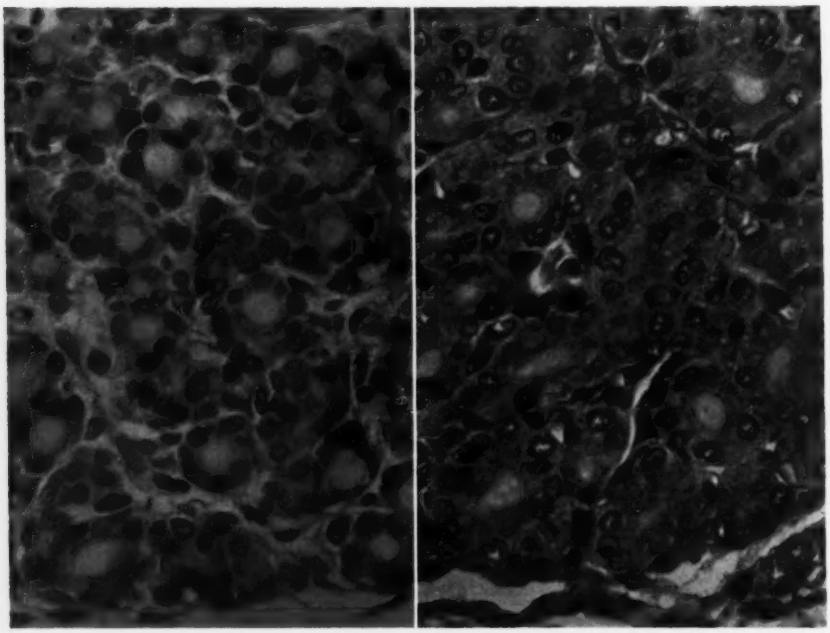

FIG. 2. (left photograph). Section of the thyroid of a control fetus of Group B. At the bottom is the region of the capsule. 3μ, iron hematoxylin, $\times$600.

FIG. 3. (right photograph). Comparable section of the thyroid of a fetus which had been given thyrotrophin (10 U. of T-Ayerst, Group C). 3μ, iron hematoxylin, $\times$600.

The effects upon the thyroid were similar to those observed in Group C, and were greatest in the fetus which had received the most thyrotrophin.

DISCUSSION

This work confirms and extends an earlier observation of histological signs of hyperactivity in the thyroids of fetal guinea pigs which had been given a single subcutaneous injection of a pituitary extract *in utero* ("hyperactivité excrétrice," Aron, 1933). Aron did not present quantitative data on the thyroids reported.[6]

[6] In chick embyros it has been reported that introduced hypophyseal extracts caused an increase in the height of the epithelium of the thyroid (Woodside, 1937).

SUMMARY

Fetuses of 19½ days (one or two per litter) were transferred from the uterus to the body cavity of the mother without interrupting the placental circulation. In one group each fetus was given a series of four subcutaneous injections of thyrotrophin. The experiments were ended at about one hour before term and 52 hours after the first injection. In another group each fetus was deprived of the hypophysis by subtotal decapitation ("hypophysectomized") prior to the first injection.

The volume of the left half of the thyroid was determined from stained serial sections by the paper-weight method. The minute structure of the right half of it was studied microscopically in thinly sectioned and specially stained preparations.

The introduced thyrotrophin increased the height of the follicular epithelium and speeded up the growth of the thyroid in both groups of fetuses. These observations show that in the rat the developing thyroid is endocrinologically labile before birth.

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INCREASED OXYGEN CONSUMPTION, AND SUCCINOXIDASE ACTIVITY OF LIVER TISSUE AFTER EXPOSURE OF RATS TO COLD[1]

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WHEN intact rats are exposed to a cold environment their consumption of oxygen increases greatly. Much of the increase may be accounted for by increased muscular activity, and direct evidence for this has been obtained. There is considerable evidence of an indirect nature which suggests that increased metabolism of non-muscular tissues may account for part of the increase. However, a direct demonstration of increased metabolic activity in visceral tissues has not yet been made. In an attempt to obtain such direct evidence, measurements of the oxygen consumption of liver slices, and of the succinoxidase activity of liver tissue homogenates, prepared from animals "acclimatized" to cold, were compared with those of normal rats.

EXPERIMENTAL

1. *Oxygen consumption of liver slices*

Two series of experiments were carried out using adult rats of the Wistar strain, bred in the Department. In the first series, two groups of 8 female rats (200 to 260 gm.) were paired on a basis of body weight. Group 1 had lived in a cold room at a temperature of $1.5° \pm 1.5°$ C. for 11 to 14 months before the experiments were started. Group 2 was selected from the rat colony maintained at $22° \pm 2°$ C. Both groups were fed Fox Breeder Cubes (Master Feeds) and water ad libitum.

Liver slices were prepared following the method described by Campbell and Davidson (1951). The slices consumed oxygen at a steady rate for at least two hours when kept in a suitable medium[2] and incubated at 37° C. The uptake of oxygen was measured manometrically according to the Barcroft-Warburg technique (Umbreit *et al.*, 1948) using oxygen as the gaseous phase.

One rat from each group was killed and liver slices from each were kept in chilled buffer medium during the preparatory period and were placed in the Warburg apparatus simultaneously. This procedure was repeated at intervals of a day or two until all the rats had been examined.

Since rats grow more slowly in the cold, the controls paired on a basis of body weight were 6 to 8 months younger than the rats acclimatized to cold. Therefore, in the second

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[1] The experiments reported in this paper form part of a project supported by the Defence Research Board of Canada.

[2] Equal portions of Locke's solution and sodium glycerophosphate (0.067 M) mixed before use, at pH 7.4.

series of experiments, 18 adult male rats were divided into two groups at the age of 4 months (body weight around 180 gms.). The animals in one group were placed in the cold room in individual metal cages for 16 days while the other group remained at room temperature for the same period. At intervals of a couple of days one rat from each group was killed, and following the same procedure as before, measurements of oxygen consumption of liver slices were made. The last pair of rats was sacrificed after exposure to cold for 36 days. In order to compare oxygen consumption on a fat-free basis, estimations of liver lipid were carried out according to the method of Best, Lucas, Ridout and Patterson (1946).

2. For the study of the effect of cold environment on succinoxidase activity, liver tissues were taken from a group of 10 rats (165 to 250 gm.) of both sexes which had been exposed to the same cold environment (1.5° ± 1.5° C.) for 27 do 70 days. Eight rats in the same range of weight maintained at room temperature constituted controls. Activity of the succinoxidase system was measured manometrically by the technique of Schneider and Potter (1943). Air, instead of oxygen, was used as the gaseous phase and the bath was kept at 38° C. The necessary cytochrome C was prepared from calf heart muscle according to the method of Keilin and Hartree (1945). Homogenization of the liver tissue was made immediately before use with the rotating glass pestle described by Potter and Elvehjem (1936). Each vessel contained 0.2 ml. of 5% liver suspension, and fortified substrate solution of the following composition: 1.0 ml. of 0.1 M phosphate buffer (pH 7.4), 0.4 ml. of 2.4×10^{-4} M cytochrome C, 0.3 ml. of 4×10^{-3} M calcium chloride, 0.2 ml. of 6×10^{-3} M aluminium chloride, 0.3 ml. of 0.5 M sodium succinate (pH 7.4), and sufficient water to make a total volume of 3 ml. In the central well of the vessel a filter paper was placed moistened with 0.2 ml. of 10% KOH. All vessels were chilled on ice and the time which elapsed during preparation, before initial readings were taken, did not exceed 30 minutes. Triplicate or quadruplicate determinations were run on each liver preparation. The activity of the succinoxidase system using sodium succinate as substrate was expressed as microliters of oxygen taken up per milligram of dry liver tissue per hour. Readings were recorded every ten minutes for a period of one hour. However, only those readings of the first 3 or 4 periods, which indicated a constant rate of oxygen uptake, were used to calculate the Q_{O_2}.

RESULTS

1. The oxygen consumptions of liver slices of 8 female rats accustomed to living in a cold environment were higher than those of 8 control animals paired on a basis of body weight (Table 1). The average Q_{O_2}, expressed as microliters of oxygen per milligram dry liver tissue per hour, was 9.19 ± S.D. 1.69 as compared to a Q_{O_2} of 7.87 ± 0.87 for the controls. The difference is significant ($P = 0.02$).

The livers of the animals exposed to cold were heavier than those of the controls (4.85 ± S.D. 0.51 gms. per 100 gms. body weight compared to 3.80 ± 0.41).

2. When comparison was carried out using male rats of comparable *age* rather than *weight*, the differences were consistently greater. The average Q_{O_2} of 9 rats which had lived in the cold for 16 to 36 days was 10.99 ± S.D. 1.04 while that of 9 controls was 8.59 ± 0.60 ($P < 0.001$). As before,

the livers of the rats which had been exposed to cold were heavier than those of the controls (4.86 ± 0.50 gm. per 100 gm. body weight compared to 3.86 ± 0.77 gms.)

Lipid content of the livers of both groups fell within the normal range, although the animals which lived in the cold had slightly higher absolute values, (5.74% of wet weight compared to 5.40%). On a basis of fat-free dry residue weight the oxygen consumption ($Q_{O_2}^{ff}$) of 7 "cold-exposed" rats

TABLE 1. OXYGEN CONSUMPTION OF LIVER SLICES OF RATS EXPOSED TO A COLD ENVIRONMENT ($2° \pm 1°$ C.)
(Q_{O_2} = c. mm. of oxygen uptake per hr. per mg. of dry tissue at 37° C.)

	Cold			Normal		
(a) Paired in body wt., all females						
Days of cold exposure	Body wt. gm.	Liver wt. gm.	Q_{O_2}	Body wt. gm.	Liver wt. gm.	Q_{O_2}
341	240	11.0	9.84	250	8.65	9.40
342	206	9.35	9.67	204	8.30	8.09
344	245	12.85	7.82	245	9.80	6.51
349	260	12.45	12.20	252	11.82	7.43
355	200	9.55	7.07	200	7.10	7.82
357	240	11.65	9.02	240	10.36	7.51
426	240	12.30	10.30	240	8.30	8.69
427	230	9.93	7.56	250	8.55	7.47
Average			9.19 ± S.D. 1.69	Average		7.87 ± S.D. 0.87
(b) Paired in age, all males						
16	195	9.60	11.56	225	7.90	8.97
17	211	9.50	11.73	220	8.00	8.84
22	227	11.70	9.73	244	10.18	8.20
23	195	8.15	9.90	253	9.05	8.70
24	232	12.52	10.27	244	9.72	8.19
27	240	10.13	11.76	278	11.30	8.85
28	160	9.00	9.92	236	9.35	8.90
31	225	10.45	9.96	292	11.96	7.34
36	222	11.15	14.11	270	10.20	9.36
Average			10.99 ± S.D. 1.04	Average		8.59 ± S.D. 0.60

averaged 14.18 as compared to a value of 10.60 for 7 controls. This difference is also highly significant ($P < 0.001$).

3. The succinoxidase activity of liver homogenate obtained from rats accustomed to a cold environment was significantly greater than that of controls. (Table 2) The average Q_{O_2} for livers of 10 cold acclimatized rats was $95.4 \pm$ S.D. 11.3 as compared to 63.0 ± 5.9 for 8 normal controls ($P < 0.001$). This indicates that a net increase of 50% was observed in the succinoxidase system of livers of rats exposed to the cold environment.

DISCUSSION

The results indicate that oxygen consumption and succinoxidase activity per unit weight of dry tissue are both significantly increased in the livers of rats which have been exposed to a cold environment for more than 16 days. If the results on oxygen consumption are calculated on the basis of total liver weight, or on the fat-free dry residue weight of liver, the observed differences became even more pronounced. The direct demonstration of these increases in metabolic activity of the liver confirms many sug-

TABLE 2. SUCCINOXIDASE ACTIVITY OF LIVERS OF RATS EXPOSED TO COLD (2° ± 1° C.)

(Q_{O_2} = c. mm. of oxygen uptake per mg. of dry tissue per hr. at 38° C.)

	Cold				Normal			
Days of cold exposure	Sex	Body wt. gm.	Liver wt. gm.	Succinoxidase Q_{O_2}	Sex	Body wt. gm.	Liver wt. gm.	Succinoxidase Q_{O_2}
27	M	235	14.10	85.0	M	240	10.30	56.1
28	F	200	10.74	100.0	F	240	9.55	63.1
35	F	190	11.65	102.0	F	196	8.25	65.1
37	F	182	11.50	82.5	F	220	10.55	68.0
41	F	196	8.70	78.2				
42	F	165	8.05	110.0	M	165	—	57.0
42	F	205	9.95	86.6	M	170	10.00	56.5
47	F	200	9.45	104.2	F	250	9.75	66.8
47	F	195	8.50	98.2				
70	F	225	9.70	107.0	F	230	7.00	71.5
Average				95.4 ± S.D. 11.3	Average			63.0 ± S.D. 5.9

gestive observations. The increase in general oxygen consumption when measured in a cold environment, the gradual increase in oxygen consumption of rats living in the cold when measurement is carried out daily at 30° C., the increase in intake of food, and of nitrogen excretion, all have suggested that oxygen consumption of visceral organs may be increased (Sellers and You, 1950).

The absolute values for succinoxidase activity of the livers of normal rats are lower than those reported by Potter (1941), and by Tipton *et al.* (1946), but are within the range reported by Elliott (1938). This difference may be accounted for at least in part by our use of air rather than an atmosphere of oxygen and by some difference in the preparations of cytochrome C. Our preparation was not a pure, salt-free extract and contained small amounts of inhibiting substances. Sodium chloride and metal contaminants have been shown to inhibit the activity of the succinoxidase system (Potter, 1941; Riley, 1950).

The mechanism of the increases observed is not entirely clear. There is evidence that feeding thyroid increases the tissue respiration of liver slices

(Gerard and McIntyre, 1933), and increases succinoxidase activity of the liver (Tipton *et al.*, 1946). During exposure to cold the thyroid gland undergoes hyperplasia (Ring, 1942) but apparently an *increased* thyroid activity is not essential for the changes observed in metabolic rate (Sellers and You, 1950).

While the action of the thyroid may be important, other hormones are involved in the adaptive changes which take place during exposure to cold and these may be concerned in the increased rate of tissue metabolism (Sellers, Reichman, Thomas and You, 1951).

SUMMARY

After rats have been exposed to a cold environment (1.5° C.) for more than 16 days, the oxygen consumption of liver slices, and the succinoxidase activity of liver homogenates is significantly increased. These findings confirm directly previous indirect evidence which suggested that the metabolic activity of visceral tissues of animals exposed to cold is increased.

Acknowledgments

The estimations of liver lipid were carried out by Drs. Jean Patterson and Jessie Ridout. The cytochrome C was prepared by Dr. E. S. Goranson. We are grateful for their help and for the advice on technical matters so freely given by Dr. James Campbell.

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THE EFFECT OF PARENTERALLY ADMINISTERED ADRENOCORTICAL EXTRACT ON THE INTRADERMAL SPREADING ACTION OF HYALURONIDASE[1,2]

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HAYES, Reed and Baker (1950) reported that prolonged direct application of an alcoholic adrenocortical extract to the skin of rats caused a marked acceleration in the spreading action of hyaluronidase, this effect being evident chiefly in the area of treatment. The concept that adrenal steroids promote hyaluronidase activity stands in contrast to the impressive array of data compiled by other investigators which demonstrate that adrenocortical extract (Opsahl, 1949 a, b, c; Winter and Flataker, 1950; Seifter, Baeder and Begany, 1949), cortisone (Winter and Flataker, 1950; Seifter *et al.*, 1950; Seifter, Baeder and Dervinis, 1949; Opsahl, 1949a) and ACTH (Seifter *et al.*, 1950) decrease the activity of hyaluronidase. There seemed to be at least two possible explanations for the contradictory findings of Hayes, Reed and Baker (1950). First, the acceleration of spreading action as revealed by prolonged local application of adrenal steroids might be an outcome of the local application technique. Second, it could result from the prolonged action of the adrenocortical steroids on the connective tissue prior to the test. If the latter explanation is correct, one should be able to demonstrate acceleration of spreading activity following prolonged parenteral administration of adrenocortical extract, if treatment with the hormone is withdrawn before the test is made. On the other hand, the injection of a large dose just prior to the test without any other pretreatment should reveal inhibition as reported by the investigators previously cited. This hypothesis is verified by the evidence to be presented in this report.

MATERIALS AND METHODS

Two experiments were planned, for which the animals were prepared as follows. *Chronic experiment:* Six litter-mate pairs of adult male Wistar rats were employed with an

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[2] The technical assistance of Mrs. A. D. Bridgeman is gratefully acknowledged.

initial weight range of 290–350 gms. Six of these animals were injected subcutaneously with 2.0 cc. of adrenal extract[3] on the abdomen once daily for 9 days and then with 1.0 cc. of the same extract twice daily for 28 days. The other 6 served as pair-fed controls, receiving the cottonseed oil vehicle of the adrenocortical extract on the same dosage schedule. Body weights were recorded weekly. *After cessation of treatment, a lapse of 2 days before study was allowed in order to reduce the amount of exogenous hormone which might remain in the circulation.*

Acute experiment: Nine litter-mates of adult male Wistar rats were employed. Control observations of the rate of spreading action were made as described subsequently. Immediately before the final observations each animal received 10.0 cc. aqueous adrenocortical extract intraperitoneally.

The technique employed for study of the spreading action of hyaluronidase has been described in detail by Hayes and Reed (1950). The hair was clipped closely from the proposed injection sites on the dorsum of the body and the animals were secured loosely for constancy of position. The animals were quieted by the subcutaneous injection of 0.3 cc. of a veterinary solution of sodium pentobarbital (6.5% solution) and were anesthetized lightly with ether only for the duration of the initial injection. The indicator employed was an isotonic solution of hemoglobin. The final injection quantity was 0.05 cc. containing 25 units of hyaluronidase.[4] The rate of injection was 0.01 cc./5 sec.

The manipulation of the injection procedure was controlled carefully and the *intradermal* injections were made in comparable sites on each side of the dorsal midline. Two studies were made on each of the chronically treated animals and 2 on each of the pair-fed controls. Two examinations were made before and 2 after the injection of the aqueous adrenocortical extract in the acute experiment.

Measurements were made of the length and width of the ellipsoidal area of spread. The first measurement, which was made at the termination of the injection was taken as a baseline. Then, observations were made every minute for 15 minutes; every 5 minutes through 30 minutes; every 10 minutes through one hour and every 15 minutes through 2 hours. Then, the increase in area for each time interval over that of the initial injection area was calculated. The arithmetic means were plotted, using the ordinate as the increase in area of spread over the initial area in sq. mm. and the abscissa as the logarithm of the time in seconds.

OBSERVATIONS

A definite loss in body weight was observed in the chronically treated animals indicating that a high degree of adrenocortical activity had been introduced by the administration of the adrenocortical extract (Fig. 1.). Paired feeding eliminated the possibility that reduced food intake might account for the weight loss. Of all observations made, 91% fell within a range of 2 sq. mm. of the plotted mean. In Figure 2, curve A shows the pattern[5] of spread in the skin of animals which had received the lipo-

[3] The adrenal extract was Lipo-adrenal Extract generously supplied by Dr. H. F. Hailman, The Upjohn Company, Kalamazoo, Michigan.

[4] The hyaluronidase was generously supplied as Alidase by Dr. Irwin C. Winter, G. D. Searle and Company, Chicago, Illinois.

[5] All spreading activity is composed of two phases: an early slow phase followed by a late or rapid phase. Such a sequence is named pattern of spreading.

adrenal extract for the long period of time; curve B is of the same study made on the control animals. It is apparent that a significant accentuation in the spreading activity of hyaluronidase has been produced by the chronic parenteral administration of adrenocortical hormones. The results are very similar to those found after prolonged local treatment with adrenocortical extract (Hayes, Reed and Baker, 1950).

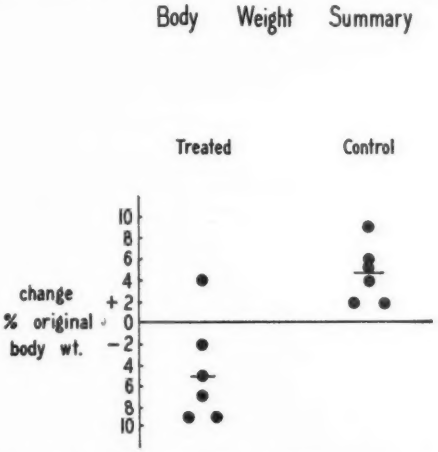

FIG. 1. Body Weight Summary. Percentage changes in body weights of the control and experimental animals. Each point represents one animal. The horizontal bars indicate the mean in each group. The control animals were pair-fed and show a mean weight gain of 5% of the original body weight; the experimental animals show a mean weight loss of 5% of the original body weight.

On the other hand, in an acute experiment in which the level of circulating adrenocortical hormones had been elevated abruptly, there was a distinctly different result. The enzyme activity had been counteracted to a large degree (curve D), closely resembling the spread of indicator without enzyme in normal skin (Hayes and Reed, 1950). Curve C represents the pattern of spread seen in the same animals without prior injection of aqueous adrenocortical extract. This curve resembles closely that previously reported for enzyme activity in normal skin (Hayes and Reed, 1950; Hayes, Reed and Baker, 1950). Again it is emphasized that under these conditions of observation the principal alteration observed in the pattern of hyaluronidase spreading activity occurs during the slow phase of spreading. The discrepancy between the patterns of spreading in the two control groups may be due to the fact that the control injections or the prolonged food restriction in the chronic experiment may have constituted a mild form of stress.

DISCUSSION

It has been demonstrated that the prolonged parenteral administration of adrenocortical hormones produces alterations which indicate a change in connective tissue ground substance, as demonstrated by increases in the rate and extent of spread induced by the enzyme hyaluronidase and modifi-

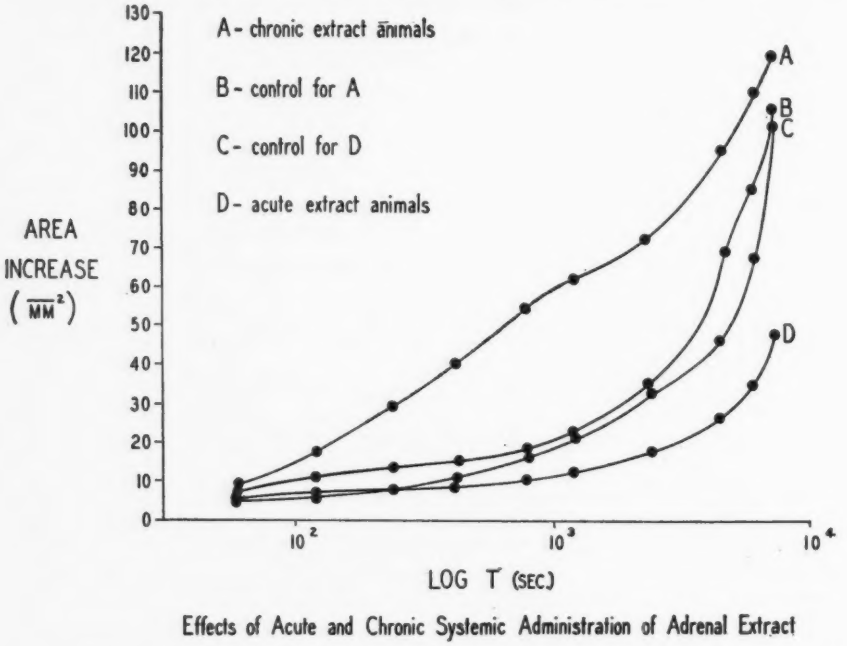

FIG. 2. Effects of Acute and Chronic Administration of Adrenal Extract. Curve A shows the rate of spread in the extract treated rats of the chronic experiment and Curve B the rate of spread in the control group. Curve D shows rate of spread in the extract treated rats of the acute experiment and Curve C, that for the controls.

cations in the characteristic pattern of the enzyme's activity. These findings duplicate rather closely the observations reported previously pertaining to the effect of local application of adrenal hormones on enzymatic spreading. When the spread of an indicator without enzyme was studied in locally pretreated skin, no essential change in the manner or extent of spreading activity was observed (Hayes, Reed and Baker, 1950). This excludes the thinness of the skin which exists under these conditions (Baker and Whitaker, 1948; Castor and Baker, 1950) as being the cause of the accelerated rate of spread. Recently, Ducommun, Timiras and Dordoni (1951) reported that cortisone inhibits the spread of hemoglobin. Their contradictory findings may have resulted from the fact that they

injected the hemoglobin subcutaneously instead of intradermally and that their observations were made at a time when the level of circulating hormones was high.

The sharp inhibition of enzyme activity by temporarily elevated levels of adrenocortical hormones shown in the acute experiment confirms the many similar reports cited earlier in this paper. The explanation for these two seemingly antagonistic effects of adrenocortical hormones may be that adrenocortical extract acts differently on each member of the enzyme-substrate complex; it is inhibitory to the enzyme (when both steroids and enzyme are present simultaneously) but with prolonged treatment, causes structural alterations in the substrate, which in turn permits more rapid spreading activity by the enzyme when it is present in the absence of high levels of adrenocortical hormones. This change in the ground substance could be essentially a reduction in amount and could have resulted indirectly from a suppression in the capacity of fibroblasts to produce it.

SUMMARY

Two effects of adrenocortical extract on the spreading action of hyaluronidase have been described. Prolonged parenteral administration prior to study with the enzyme produced a marked increase in the rate and extent of spreading and a significant alteration in the pattern of spreading; acute elevation of the circulating level of adrenocortical hormones, without other pretreatment, markedly inhibits the spreading action of the enzyme. The first effect is interpreted as being the result of an alteration in the physical state or amount of the ground substance; the second, as resulting from a direct inhibitory action on the enzyme itself.

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LOCAL MAINTENANCE OF THE RABBIT CORPUS LUTEUM WITH OESTROGEN

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IT HAS recently been shown (Höhn and Robson, 1950) that when small crystals of various oestrogens are implanted into the corpus luteum of pseudo-pregnant rabbits and the animals then hypophysectomised, there was no selective maintenance of the implanted corpus luteum. Depending on the oestrogen actually used and on the dose, the corpora lutea in both ovaries were either equally maintained or degenerated to the same extent. This suggested that oestrogens did not act directly on the luteal cells but through some indirect mechanism.

However, evidence obtained by one of the authors of the present paper (J. H.) indicated quite clearly that the implantation of stilboestrol dipalmitate into the corpus luteum produced local maintenance in non-hypophysectomised animals. These results were privately communicated to J. M. R. and led him to perform the experiment with the same oestrogen in animals which were hypophysectomised after the implantation of the oestrogen. The present paper presents the combined results on the effects observed both in normal and in hypophysectomised rabbits.

MATERIAL AND METHODS

For implantation of corpora lutea two methods of preparing implants of suitable size were used. None of these implants have been weighed: their size is estimated at 50–500 μg. each. *Chips* have been used in a few cases: they were prepared by gently fusing the material in a watch glass, allowing it to solidify, and then breaking it up into triangular laminae. *Cylinders* were prepared by dipping pieces of drawn-out glass tubing into the fused substance; this, when solidified, was easily pushed out of the slightly tapered tubing, and broken with a razor blade into convenient lengths.

Cholesterol was used as an excipient in some cases. Weighed amounts of the two substances were mixed and fused together. Triphenylchlorethylene was much more easily prepared as cylinders if a little (5% or less) tallow was added as excipient. Single crystals of this substance have also been used for implants.

Great care has to be taken in the implantation, otherwise the trauma may by itself lead to degeneration of the implanted corpus luteum, presumably owing to interference with its blood supply.

It is not possible to determine by purely macroscopic examination whether an implant has had a local effect on a corpus luteum. Serial sections have therefore been cut in such cases until the implant has been located.

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The rabbits were kept in separate cages. Pseudo-pregnancy was induced by intravenous injection of chorionic gonadotrophin (P.U.). Implants into corpora lutea of *chips* were made simply by pushing the chip, held in fine forceps, into a hole made in the surface of the corpus. If the hole was not made too large there was little trouble with subsequent extrusion of the chip. *Cylinders* were more easily flushed back through the hole as blood oozed through the wound. The technique of their implantation was, however, much more simple.

The cylinders were placed in the end of short pieces of drawn out glass tubing, held in position by a small drop of water. This tube was held against the hole made in the corpus, and the cylinder pushed into it with a wire plunger passed down the tube.

Hypophysectomy was performed by the method of Firor (1933) and the completeness of the operation checked at post-mortem examination.

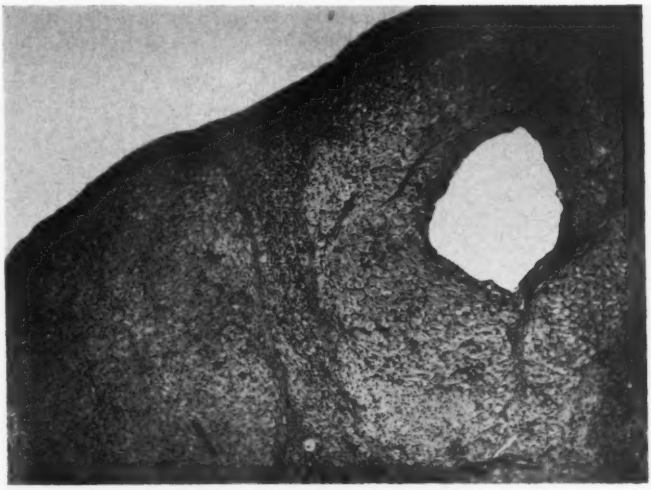

FIG. 1, A. Microphotograph of implanted and adjacent corpus luteum of rabbit 430 (8 days after complete hypophysectomy) showing maintenance around implant and degeneration at periphery of implanted corpus luteum and in adjacent (non-implanted) corpus luteum. ×40.

RESULTS

1. *In non-hypophysectomised animals*

Implants were made 4–9 days after the ovulating gonadotrophin injection (50 I.U.) and the corpora examined when 26–39 days old.

Stilboestrol dipalmitate was used in 4 animals, 1–4 corpora lutea being implanted. In each case the treated corpus luteum persisted, and the effect was localised to implanted corpora lutea, other corpora in the same ovary being degenerate. Moreover, the local nature of the effect was detectable without reference to control corpora, for it was graded within the im-

planted corpus which was unevenly maintained: The part adjacent to the implant was well maintained while more peripheral parts were less well maintained or had degenerated to the same extent as in control (non-implanted) corpora lutea. Such an effect has also been obtained with other oestrogens (see below). Sometimes a difference in nuclear size was more obvious than a difference in cell size. One animal had one corpus implanted with a chip of the dipalmitate and another corpus with the di-n-butyrate: local maintenance was obtained with both. Another animal showed a rather interesting effect: A cylinder of stilboestrol dibutyrate was (though not

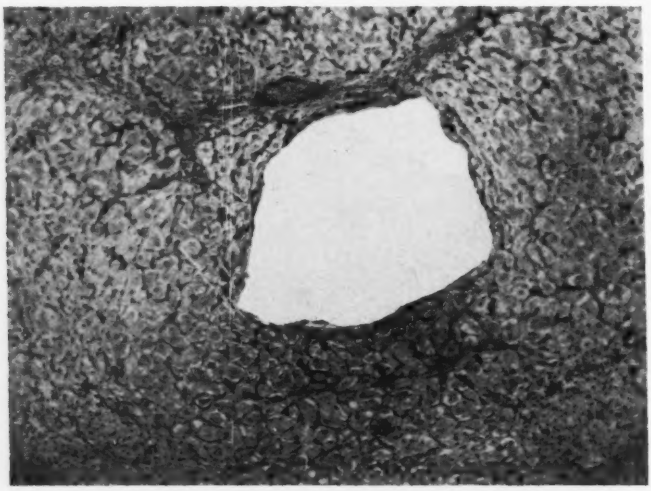

FIG. 1, B. Higher magnification of area around implant. ×75.

deliberately) implanted just between two adjacent corpora lutea; there was maintenance of about half of each.

Two single implants were tried with unesterified stilboestrol. In one case all the corpora lutea were maintained; in the other the chip implanted was probably completely absorbed before killing, and all the corpora lutea were small and white; the ovary was not examined further.

The effect with triphenylchlorethylene (2 animals) was restricted to a very narrow zone around the implant. One animal was implanted with a single crystal, the other with a cylinder.

Implants have also been made into corpora of pregnancy 23–25 days after mating. In B 26, 7 of 9 corpora lutea were implanted with cylinders of triphenylchlorethylene. The animal was killed 13 days later, having some time previously given birth to and eaten its young. A local effect on the

corpora was produced, but not very narrowly restricted within the treated corpora—a result suggesting a summation of local and general oestrogenic effects.

One pregnant female was implanted with a single cylinder of free stilboestrol and killed 36 days after mating. There was general maintenance of the corpora, though the 7 young, retained in utero, had been dead for some days.

Other pregnant females were treated as follows: B 19: 3 corpora were implanted with chips of dipalmitate; the animal gave birth to young at 32 days and was killed at 39 days; B 67: 2 corpora were implanted with cylinders of dibutyrate; young born at 32 days, animal killed at 36 days; in 3 animals single corpora were implanted with fused oestrogen—cholesterol cylinders, as follows: B 69, 10% oestradiol, young born at 31 days, killed at 36 days; B 71, 47% stilboestrol, young born at 32 days, killed at 36 days; B 72, 50% oestrone killed at 35 days, young born and eaten sometime previously. The others reared their young.

In all these there was maintenance of the treated corpora and not of controls. In B 67 a graded effect was detectable in only one of the two implanted corpora lutea: in the rest the effect was localised within the corpora.

2. *Hypophysectomised rabbits*

The details of the experiments on such animals are given in Table 1. Ovulation was induced in these animals by the intravenous injection of about 400 I.U. of chorionic gonadotrophin and the interval between this and the implantation was always 10 days. Hypophysectomy was always performed on the day following implantation. Only one corpus luteum was implanted in each animal with a pellet of stilboestrol dipalmitate weighing about 0.5 mg.

TABLE 1. DETAILS OF EXPERIMENTS ON HYPOPHYSECTOMIZED ANIMALS. IN ALL THESE THE INTERVAL BETWEEN THE OVULATING INJECTION (ABOUT 400 UNITS OF CHORIONIC GONADOTROPHIN) AND THE IMPLANTATION WAS 10 DAYS AND HYPOPHYSECTOMY WAS PERFORMED ON THE DAY FOLLOWING IMPLANTATION. ONLY ONE CORPUS LUTEUM WAS IMPLANTED IN THESE ANIMALS, IN ALL CASES WITH STILBOESTROL DIPALMITATE

Animals No.	Interval between hypophysectomy and postmortem days	Effect on implanted corpus luteum	Hypophysectomy
418	6	Local maintenance	Complete
422	8	Local maintenance	About 2/5ths of the gland left
424	13	Local maintenance	Complete
429	8	Local maintenance	Almost half the gland left
430	8	Local maintenance	Complete
433	14	Local maintenance	Complete

It will be seen that local maintenance of the implanted corpus was produced in all the animals. All the other corpora lutea in the implanted ovaries (with one exception) had degenerated. Sections (though not serial sections) were cut of the non-implanted ovaries and all the corpora lutea seen had degenerated. The exception mentioned above was in animal 433 and rather beautifully further illustrates the local action of the oestrogen: The implant was placed rather deep into a corpus luteum and very near to another corpus luteum immediately adjacent to the implanted one. There was maintenance of luteal tissue around the implant not only in the implanted corpus luteum but also in the contiguous tissue of the adjacent corpus luteum.

DISCUSSION

The results show quite clearly that an oestrogen, implanted into the corpus luteum, can maintain the morphological structure of part of the corpus luteum around the site of implantation and not maintain other corpora lutea not directly affected by the oestrogen diffusing from the implant. The oestrogen can thus act directly on the luteal tissue and does not need to undergo some change in the body before being capable of producing this effect. This finding is similar to that obtained when the actions of oestrogens are investigated on several other tissues e.g. the vagina (Robson and Adler, 1940).

It must be noted, however, that there is no evidence that luteal cells directly stimulated by oestrogen exert any endocrine activity and secrete progesterone. It is, nevertheless, probable that this secretory activity does occur, since corpora lutea maintained by the systemic administration of oestrogen are active and can maintain pregnancy in hypophysectomized rabbits (Robson, 1939).

The previous negative results on the local action of oestrogen on the corpus luteum (Höhn and Robson, 1950) emphasise that the margin between local and systemic effects is small. Presumably the oestrogen will produce the local effect only if it can diffuse very well from the site of implantation and yet be absorbed quite slowly into the general circulation.

SUMMARY

Corpora lutea in pseudo-pregnant and pregnant rabbits were implanted with chips or cylinders of various oestrogens and the animals examined at various periods after implantation. Implanted corpora lutea were maintained well beyond their normal life span.

Corpora lutea in pseudo-pregnant rabbits were implanted with oestrogen and the animals then hypophysectomised. Local maintenance of the implanted corpora lutea was observed.

We should like to express our thanks to Messrs. L. A. Westwood, J. Pike and D. Canwell for much tedious preparation of histological material and to the Medical Research Council for a grant which defrayed part of the expenses of this investigation.

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THE ROLE OF ADRENAL STEROIDS AND ACTH IN GLUCONEOGENESIS*

A STUDY IN PHLORIDZINIZED ANIMALS

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STUDIES in phloridzinized animals were responsible for some of the earliest indications that the adrenal steroids play an important role in the regulation of gluconeogenesis (Wells, 1940, and Wells and Kendall, 1940). Unfortunately, at the time of these early studies, no good preparations of adrenocorticotrophic hormone (ACTH) were available. Experiments assessing the effectiveness of these steroids on ketone excretion were also performed but usually in animals other than those used in the glucose and nitrogen studies and under somewhat different experimental conditions (Wells and Kendall, 1941). The present investigation was undertaken in an attempt to evaluate the role of the adrenal in gluconeogenesis by administration of individual adrenal steroids to adrenalectomized phloridzinized rats and observation of their effects in graded doses upon glucose, nitrogen and ketone excretion. This was also done in intact animals given ACTH.

MATERIALS AND METHODS

Young mature male rats of the inbred Fischer strain, bred in our laboratories, weighing from 125 to 225 gm. were used. The materials employed and their sources are given in Table 1.

Bilateral adrenalectomy was performed in a single sitting through one cutaneous incision after the animals had been anesthetized with ether. For two days following adrenalectomy the rats were hopper fed Purina Laboratory Chow and tap water *ad libitum;* food was removed on the third day. On the second day of fasting, the rats were injected subcutaneously with 50 mg. phloridzin suspended in 1 cc. olive oil and the experimental amount of steroid either dissolved or suspended in 1 cc. sesame oil. The alcoholic solution of hog adrenal extract was added to sesame oil and the alcohol removed *in vacuo.* Both the intact and adrenalectomized controls were given 1 cc. sesame oil as well as the 50 mg. phloridzin in olive oil. One group of intact animals was given varying amounts of ACTH suspended in water and injected in two doses 6 hours apart. One cubic centimeter of sesame oil and the usual amount of phloridzin were given with the

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first injection of ACTH. At least 6 animals were included in each experimental group, unless a large proportion of the animals died during the collection period.

Immediately after the injection and weighing, the rats were placed in individual metabolic cages in glass funnels and urine was collected under mineral oil with sodium fluoride as a preservative. Feces and urine were separated by a marble in the neck of the funnel. At the end of 24 hours, the cages of surviving animals were washed down with water and the urine and washings diluted to 100 ml.

Glucose was determined by Benedict's quantitative method (Osgood, 1940). Nitrogen

TABLE 1*

Material	Source	Supplied by
Kendall's Compound A acetate (11-dehydrocorticosterone acetate)	Synthetic	Dr. J. M. Carlisle, Merck
Kendall's Compound B (corticosterone)	Adrenal perfusion	Dr. G. G. Pincus, Worcester Foundation
	Natural	Dr. William Haines, Upjohn
Kendall's Compound F (17-hydroxycorticosterone)	Natural	Dr. William Haines, Upjohn
		Dr. Harold Mason, Mayo Foundation
Reichstein's Compound L Acetate	Synthetic	Dr. George Rosencrantz, Syntex
Reichstein's Compound P Diacetate	Synthetic	Dr. George Rosencratnz, Syntex
Reichstein's Compound S Acetate	Synthetic	Dr. Percy Julian, Glidden
		Dr. C. R. Scholz, Ciba
11-desoxycorticosterone acetate	Synthetic	Mr. F. E. Houghton, Ciba
Cortisone acetate (11-dehydro-17-hydroxycorticosterone acetate)	Synthetic	Dr. J. M. Carlisle, Merck
Cortisone (11-dehydro-17-hydroxycorticosterone)	Synthetic	Dr. A. Gibson, Merck
Δ_5-pregnenolone acetate	Synthetic	Dr. Ernst Oppenheimer, Ciba
21-acetoxy-Δ_5-pregnenolone	Synthetic	Dr. Ernst Oppenheimer, Ciba
Progesterone	Synthetic	Dr. George Rosencrantz, Syntex
Testosterone	Synthetic	Dr. Ernst Oppenheimer, Ciba
Hog Adrenal Extract	Hog adrenals	Dr. William Haines, Upjohn
ACTH (160% activity of La-1-A)	Hog pituitaries	Dr. E. E. Hayes, Armour
Phloridzin		Dr. E. C. Reifenstein, Ayerst

* We would like to thank the men mentioned for generous supplies of material.

was determined by semi-micro Kjeldahl analysis, concentrated sulfuric acid and hydrogen peroxide being used in the digestion. Distillation was accomplished according to the method of Ma and Zuazaga (1942). Acetone bodies were precipitated by the Van Slyke method (Van Slyke, 1917) and the mercury in the precipitate was determined colorimetrically, with the Coleman junior spectrophotometer at wave-length 550 mμ as described by Crandall (1940). Glucose, nitrogen, and ketone values were calculated as mg. per 100 gm. rat, and the average values for each experimental group were plotted against the amount of steroid administered.

RESULTS

Intact rats, injected with phloridzin on the second day of fasting, excreted during that 24-hour period an average of 668 mg. glucose, 215 mg. nitrogen, and 31.4 mg. ketones. Adrenalectomy two days prior to the fasting period caused the average excretion values to drop to 50 mg. glucose and 16.6 mg. nitrogen, with ketonuria completely inhibited. Administra-

tion of adrenal steroids at the same time as the phloridzin effected partial restoration of glucose, nitrogen, and ketone excretion. The effects of the various compounds used are summarized in the accompanying graphs. In none of the doses of steroids employed in this study was it possible to attain

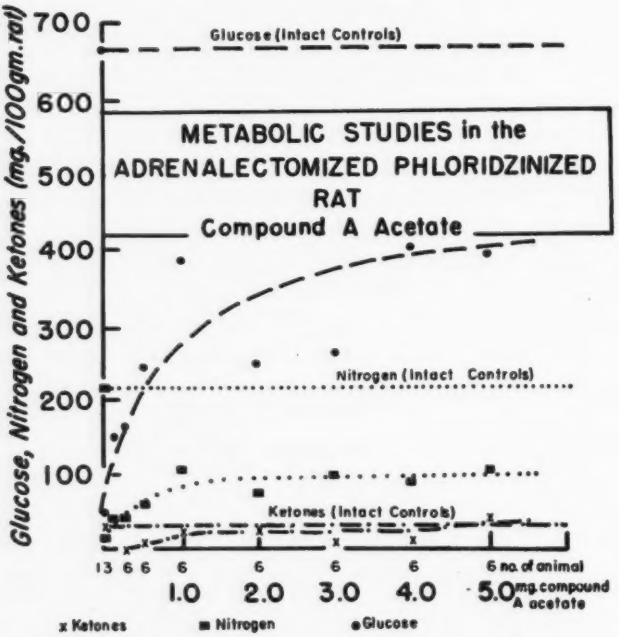

FIG. 1. Glucose, nitrogen, and ketone excretion in the adrenalectomized rat following administration of Compound A acetate.

the excretion of glucose or nitrogen that was found in the intact phloridzinized animals. Ketone output, however, was raised to a level above that of the intact controls in those animals receiving Compound A acetate, hog adrenal extract, corticosterone, and Compound F.

Compound A acetate was found to be the most effective in raising glucose excretion (Fig. 1). Although the initial rise in nitrogen excretion followed parallel to that of glucose, it tended to level off somewhat more than the glucose. The urinary excretion of ketones by animals given 5 mg. of Compound A acetate reached a level slightly above that seen in intact controls.

Hog adrenal extract also showed a steeply rising curve of glucose excretion accompanied by an increase in urinary nitrogen (Fig. 2). There was

an early sharp rise in ketones which reached a maximum level with 0.5 ml. of extract (equivalent to 1 mg. cortisone) and then with increasing doses of extract fell off until at 2 ml. of extract it had returned almost to the base line. This occurred despite the continued high glycosuria and urinary nitrogen excretion.

Corticosterone or Compound B was not as effective as Compound A actate or hog adrenal extract in raising the urinary glucose (Fig. 3). Again

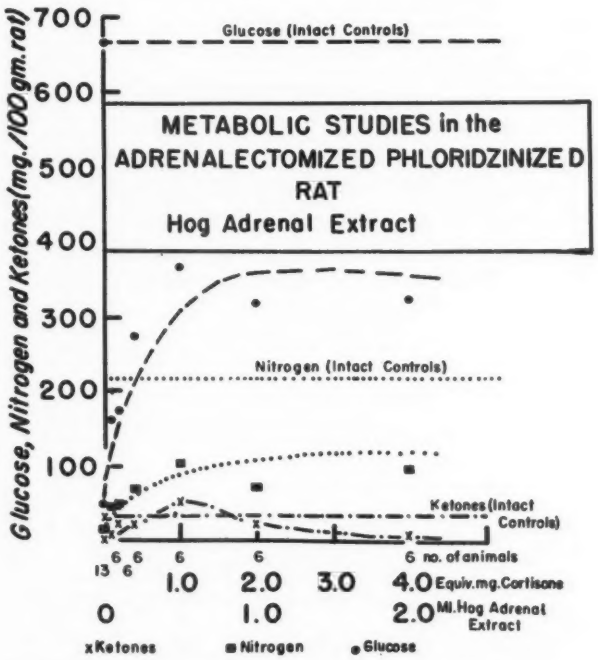

FIG. 2. Glucose, nitrogen, and ketone excretion in the adrenalectomized rat following administration of hog adrenal extract.

the lower part of the curve was accompanied by rising excretion of nitrogen and, at higher levels, by a slowly rising excretion of ketones.

The curve for the effect of cortisone on glucose excretion did not rise as steeply as that for corticosterone (Fig. 4), but it reached approximately the same maximum. The nitrogen curves for the two were practically identical; cortisone, however, had much less effect on ketonuria than any of the compounds having considerable effect on glycosuria.

Figure 5 shows the results obtained with Compound F (17-hydroxy-

corticosterone). An initial sharp rise in the curves for glucose and nitrogen is followed by a flattening out at the point where the ketone curve begins to rise. It is of interest to point out that the curves for glucose and nitrogen with Compound F show the steepest rise of any of the compounds studied, being close to maximum excretion values at 250 μg.

Cortisone acetate was much less effective than free cortisone or any of the other substances mentioned in raising urinary glucose and nitrogen

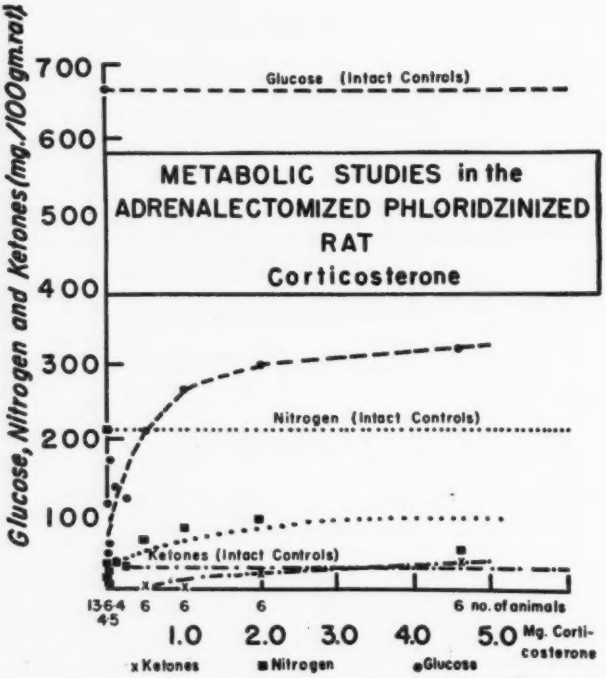

FIG. 3. Glucose, nitrogen, and ketone excretion in the adrenalectomized rat following administration of corticosterone.

(Fig. 6). As with cortisone, only minimal amounts of ketone were found, even on amounts as great as 10 mg. of cortisone acetate per animal. An essentially similar pattern of excretion was obtained with desoxycorticosterone acetate (Fig. 7). The main difference observed between the two is that at 5 mg., the ketone excretion with desoxycorticosterone acetate was somewhat greater than that with cortisone acetate.

The remaining compounds, progesterone, Δ_5-pregnenolone acetate, Δ_5-

21-acetoxypregnenolone, testosterone, Compound L acetate, Compound P acetate, and Compound S acetate were all essentially ineffective at the dosage level employed. In Figure 8, the results with Compound S acetate, the most effective of this group, are presented. The differences observed here do not appear to be really significant.

It is of great interest that with all the compounds studied, with the exception of desoxycorticosterone acetate and the hog adrenal extract, the

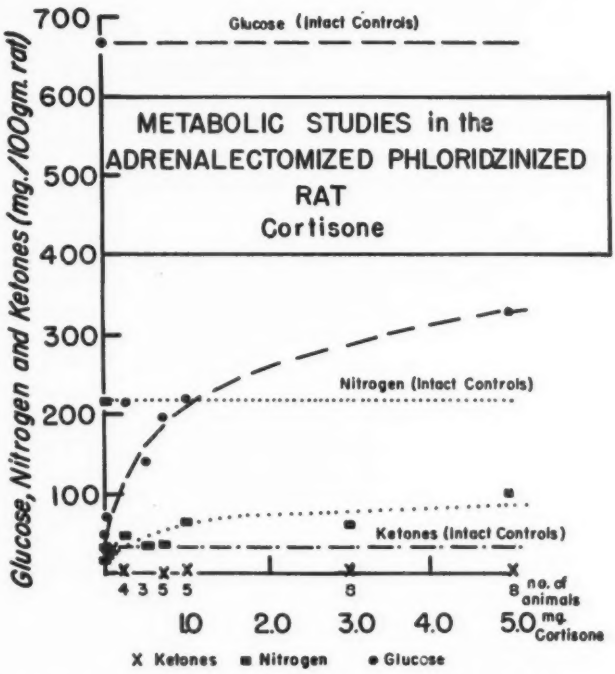

FIG. 4. Glucose, nitrogen, and ketone excretion in the adrenalectomized rat following administration of cortisone.

small doses of the active steroids seemed to be toxic to the animal in some manner; that is to say, of the animals receiving dosages of steroids not great enough to produce a significant increase in glucose excretion, more died during the urine collection period than did untreated adrenalectomized animals. Of the relatively ineffective steroids mentioned, in the preceding paragraph, even large doses, 5 or 10 mg. per animal, failed to protect the animal and showed this anomalous effect of more animals dying than if they were not treated at all.

Figure 9 shows the results obtained from ACTH treatment of intact animals. The doses of ACTH are expressed as mg. of equivalence to Armour's standard La-1-A. Small amounts (1–2 mg.) of ACTH caused a great decrease in glycosuria and urinary nitrogen, but increasing amounts up to

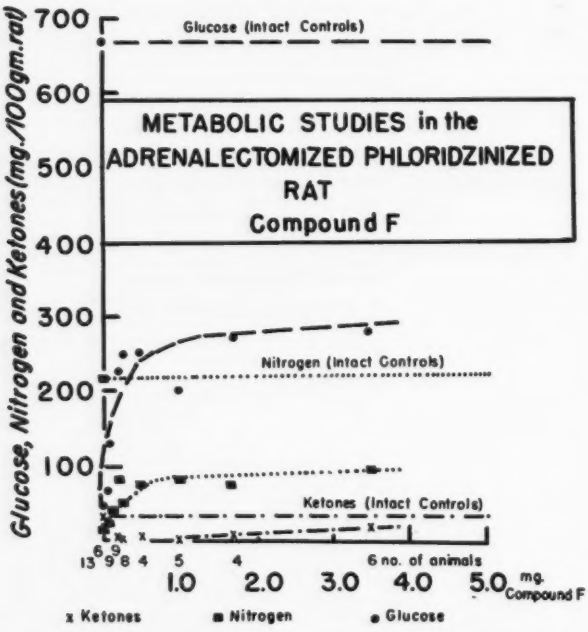

FIG. 5. Glucose, nitrogen, and ketone excretion in the adrenalectomized rat following administration of Compound F.

40 mg. led to an increase in urinary glucose. The highest urinary glucose level, 911 mg. per 100 gm. rat, was attained with 40 mg. of ACTH. Despite this great increase in glycosuria there was no accompanying increase in urinary nitrogen; even at 40 mg. of ACTH the urinary nitrogen level had not returned to that of untreated intact animals. This increase in glucose unaccompanied by an increase in nitrogen led to substantially higher G/N ratios than those observed in any other group of animals. It is of further interest to note that all doses of ACTH employed caused increasing excretion of urinary ketones, reaching three times that of the controls at 40 mg. of ACTH.

Adrenalectomized rats treated with ACTH in doses of 10 mg. and 40 mg. failed to survive the collection period.

DISCUSSION

There are several theoretic results which can be discussed from the data presented. Of substantial interest is the fact that within the range of doses employed in this study there was a tendency for both the glucose and nitrogen excretion curves, particularly the latter, to reach a plateau, and it

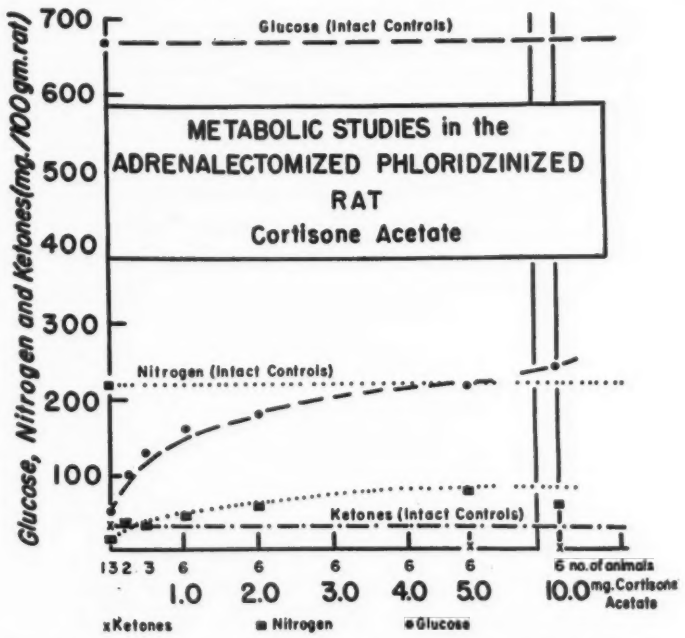

FIG. 6. Glucose, nitrogen, and ketone excretion in the adrenalectomized rat following administration of cortisone acetate.

was often at this point that the ketone curve began to rise. With the exception of only 3 animals, ketonuria was not present unless glucose excretion was greater than 150 mg. per 100 gm. animals.

Our data are in agreement with those of Mirsky and co-workers (1940) in that we found a great decrease in ketonuria as the invariable accompaniment of adrenalectomy. They suggested that these changes are due to the general lower metabolic activity of the adrenalectomized animal.

It would appear obvious that in the adrenalectomized animals where the increased urinary glucose is accompanied by an equivalent increase in urinary nitrogen this glucose is coming from protein by gluconeogenesis. One must speculate, however, upon the source of the additional glucose

in instances where it connot be accounted for by additional nitrogen, as in the case of the animals treated with ACTH and the more effective steroids. Since these larger amounts of glucose have usually been accompanied by a perceptible increase in urinary ketones, it is possible, of course, that the greater amount of available ketone has inhibited peripheral utilization

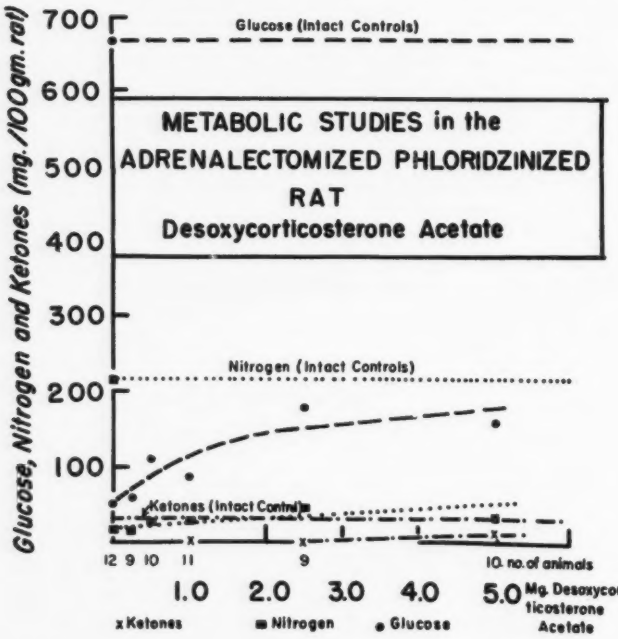

FIG. 7. Glucose, nitrogen, and ketone excretion in the adrenalectomized rat following administration of desoxycorticosterone acetate.

of glucose. It is also of interest to speculate as to the source of this increased urinary ketone. Since animals fasted for 24 hours have substantially depleted their liver glycogen stores, and since the increase in glucose is unaccompanied by a further increase in urinary nitrogen, this leaves us with body fat as the most likely source of the ketones, and of the increased carbohydrate. It thus appears that the urinary ketones and also directly or, as just suggested, indirectly (through the ketone suppression of peripheral tissue utilization of glucose) some of the urinary glucose must have arisen from the body fat.

Certain quantitative differences exist between our experiments and those reported by Wells and Kendall. These can probably be explained by the

variation in material and procedure between their various experiments and ours.

SUMMARY

Adrenalectomy leads to a large decrease in the glucose and nitrogen excretion, and complete suppression of ketone excretion, in fasting phloridzinized rats.

Excretion of glucose and nitrogen can be elevated by administration of various 11-oxygenated steroids and desoxycorticosterone acetate, but there has been no instance in which the glucose or nitrogen under the conditions of our experiments has been raised much above half of that seen with intact controls. With several compounds, however, it is possible to raise the ketone excretion above that of the intact controls.

ACTH in large doses leads to an increase in urinary glucose unaccompanied by an increase in nitrogen but accompanied by a substantial increase in ketones.

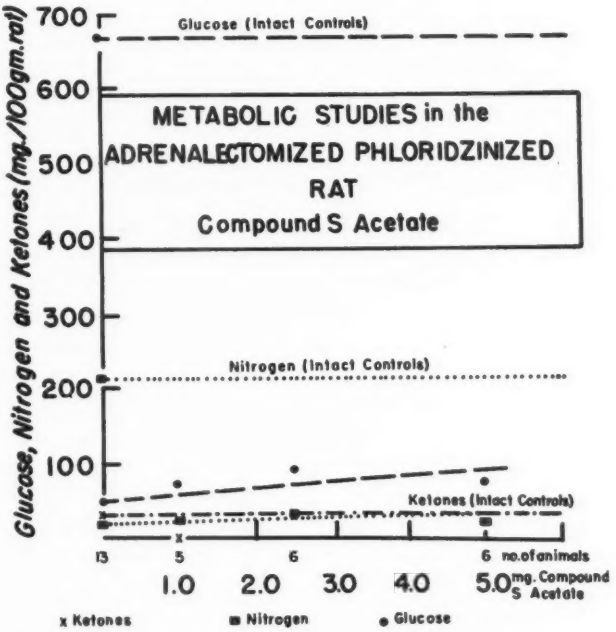

FIG. 8. Glucose, nitrogen, and ketone excretion in the adrenalectomized rat following administration of Compound S acetate.

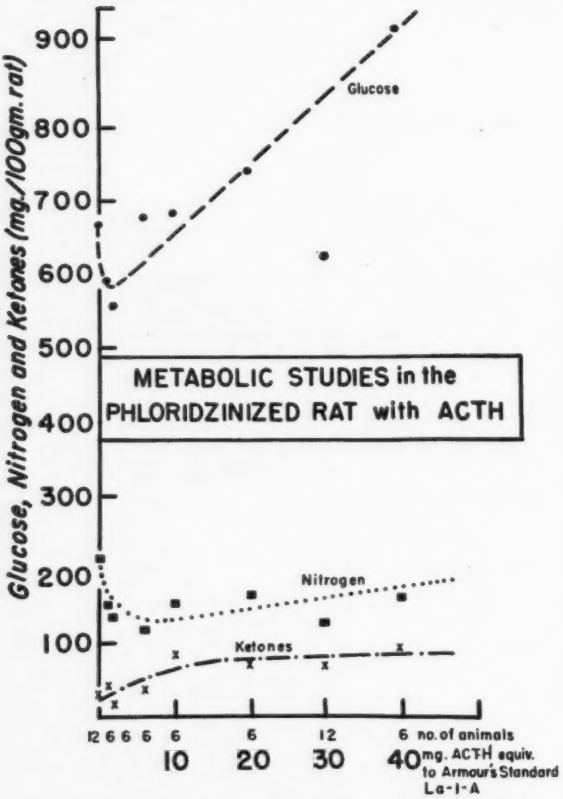

FIG. 9. Glucose, nitrogen, and ketone excretion in the intact rat following administration of ACTH.

These results are interpreted as indicating that the adrenal not only has an effect on gluconeogenesis from protein but also on fat breakdown.

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TWENTY-FIVE YEARS OF RESEARCH ON THE BIOCHEMISTRY OF THE THYROID GLAND[1]

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IT MUST now be about 20 years since my teacher and friend George Barger spoke in this Institution on the early work on the chemistry of throxine which had been done in my laboratory and in which he and I had been associated. When I received the honour of an invitation to deliver this Richardson Lecture, it seemed to me appropriate that I should use the occasion for a review of the progress of work on the thyroid problem from the point where Barger left off. Naturally I shall treat the subject almost entirely from the biochemical point of view, for it is this aspect of the work with which I have been partly concerned and which I have followed most closely; also I must begin my story a little earlier than the date that I have mentioned.

The discovery of thyroxine by Kendall in 1915, followed a little more than ten years later by the elucidation of its chemical constitution and by its synthesis (Harington, 1926; Harington and Barger, 1927), was the culmination of the researches of many different types of investigators spread over a period of centuries. Indeed the story of research on the thyroid gland up to this point offers, as I have emphasised many times, one of the most striking examples of that blending of different scientific disciplines to the solution of a medical problem which we recognise as an outstanding characteristic of medical research today.

Nevertheless, the discovery of thyroxine and its chemical identification, although of the greatest scientific interest, had rather surprisingly little immediate impact on the thyroid problem viewed as a whole. At the time when these discoveries were made there already existed a large body of knowledge regarding the general physiological functions of the thyroid gland. The fact that the gland secreted an active principle which was essential for the maintenance of normal metabolism had been recognised since the work of Kocher on the effects of surgical removal of the thyroid and the almost simultaneous recognition of myxoedema as a condition resulting from failure of thyroid function. The control of myxoedema by replacement therapy with thyroid gland had been established medical practice for 30 years or more. Moreover it was the generally accepted view

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[1] Richardson Lecture, delivered at the Massachusetts General Hospital, Boston, May 16, 1951.

that normal thyroid function was intimately wrapped up with the element iodine. This view was based on several lines of evidence; on the biological side there was the curative effect of iodine on certain forms of goitre, the association of endemic goitre and cretinism with environmental deficiency of iodine and the experimental work of Marine that showed the almost quantitative relationship between structure of the thyroid gland and its content of iodine; on the chemical side there was the demonstration, first made by Baumann who discovered iodine in the thyroid gland and confirmed by later workers, that the iodine-containing substances in the gland were those that had the characteristic effects on metabolic processes.

Taking all these facts into consideration therefore no-one could entertain serious doubt that the active principle of the thyroid gland when it was isolated would prove to be an iodine-containing compound, and to this extent the discovery and identification of thyroxine might be regarded merely as an interesting objective scientific demonstration of the truth of views already long held, and as having little further importance. This impression would be reinforced by the fact that the availability of thyroxine in the pure state offered no therapeutic advantage; indeed the physical and chemical properties of thyroxine are such that for therapeutic purposes whole dried thyroid gland or relatively crude extract has so far almost always been preferred to the pure hormone. It is true that this picture may change since a group of workers in England (Hart and Maclagan, 1950) have recently claimed good results from the oral administration of the sodium salt of thyroxine, which can now be manufactured in quantity by a relatively simple and inexpensive synthesis from tyrosine (Chalmers *et al.*, 1949); if these results receive confirmation from other sources, it may be that pure thyroxine will eventually compete as a therapeutic agent with the easily available and readily standardised thyroid gland.

There might therefore be some excuse for the view that the acquisition of full knowledge of the chemistry of thyroxine represented an end-point in research. Such a view however, though excusable, would have been wrong for the reason that there can in principle be no such thing as an end-point in research. Each new discovery is likely to raise at least as many questions as it answers, and the work on thyroxine has proved to be no exception to this general rule. Much of the newer work on the thyroid gland to which I am going to refer today will already be familiar to you—much of the most important of it has indeed been done in this city—but my hope is to present you with a fairly comprehensive picture of the developments which have occurred and to indicate how these have in large measure depended on our knowledge of thyroxine in a way that could not have been foreseen.

The isolation of thyroxine and the proof of its chemical constitution im-

mediately raised two points of interest to physiologists and biochemists. In the first place the yield of thyroxine obtainable from the thyroid was, even under the best conditions, so low in relation to the total iodine content as to suggest the presence in the thyroid of other iodine-containing compounds; the iodine remaining after all possible thyroxine had been extracted was for the most part in organic combination although it was not associated with any demonstrable biological activity; the possibilities existed that the non-thyroxine iodine-containing components of hydrolysates of thyroid gland were artefacts formed from thyroxine by the hydrolytic process or that they were already present in the original gland material. A study of the chemical properties of thyroxine rendered the first of these possibilities unlikely, leaving as the greater probability a more interesting situation in which thyroxine was accompanied in the thyroid gland by other organic but physiologically inactive iodine compounds.

Such compounds were on general grounds likely to be chemically related to thyroxine and it was not unreasonable to suppose that they might be concerned in its biogenesis; the question of the probable nature of the non-thyroxine iodine of the thyroid thus at once became closely associated, in thought on the general problem, with the second question raised by the chemical work on thyroxine, namely the mechanism by which the latter might be formed in the body.

As a matter of fact this last question had already been considered before the chemical constitution of thyroxine was known with certainty, and as so often happens knowledge gained by earlier workers provided the necessary lead to its solution. Once the nature of the iodine-free skeleton of thyroxine—the substance that we now know as thyronine—was ascertained, it was clear that thyroxine was closely related to tyrosine. It happened that at this time the only naturally occurring iodine-containing amino-acid known was 3:5-diiodotyrosine; this compound had been isolated many years previously from natural sources; it could be synthesised by the action of iodine on tyrosine and it was also known to be formed when proteins containing tyrosine were treated with iodine under appropriate conditions. It did not therefore require a great stretch of imagination to develop the theory that thyroxine was formed in nature from tyrosine through the stage of diiodotyrosine; such a mode of formation would require the coupling of two molecules of diiodotyrosine with loss of one side-chain and the formula of the compound which would result from this process could be deduced.

In the absence of any conclusive chemical evidence of the positions occupied by the iodine atoms in the molecule of thyroxine, it was mainly upon the above-mentioned deduction that the successful synthesis of thyroxine was planned and carried out. The very success of the synthesis

constituted in my mind strong evidence in support of the biogenetic hypothesis on which it had been based; clearly however this evidence was far from conclusive and it is only in recent years that the truth of the theory has been completely established.

It was at once apparent that if the hypothesis were correct it was extremely probable that thyroxine would be accompanied in the thyroid gland by the diiodotyrosine which was its supposed immediate precursor. Here therefore was a lead which could be followed in the examination of the non-thyroxine iodine of the thyroid. Pursuit of this idea led without great difficulty to the isolation of diiodotyrosine from the non-thyroxine fraction (Harington and Randall, 1929). Proof of the existence of diiodotyrosine in the thyroid having been obtained, a serious attempt was made to work up the iodine-containing compounds quantitatively in order to see whether anything of this nature other than thyroxine and diiodotyrosine was present.

The methods then available were limited in scope; nevertheless by the most careful fractionation process that could be carried out, with analytical control of every step, evidence was obtained which showed without doubt that thyroxine and diiodotyrosine between them accounted for the greater part of the iodine in the thyroid. Indeed at that time and for many years afterwards, I regarded the evidence as sufficient to exclude the occurrence of other iodine-containing organic compounds. It has required the application of modern methods of chromatographic analysis, with their great power of resolution and the devastating light that they throw on the homogeneity of supposedly pure compounds, to show that I was wrong in this assumption; the fact remains true that the major iodine-containing compounds of the thyroid are thyroxine and diiodotyrosine; others are however also present. Thus [from the work of Fink and Fink (1948) and Taurog *et al.* (1949)] we now know that a significant proportion of the total iodine may be represented by monoiodotyrosine, although the figure of 15% which the latter authors give is probably too high, and there are further components which occur in minor amounts but which may turn out to be of interest from the point of view of the metabolism of thyroxine. At the moment however I am not concerned with these minor components but only to point out the further support for the biogenetic hypothesis that was provided by the simple demonstration of diiodotyrosine in the thyroid gland.

Additional circumstantial evidence of quite a different character could theoretically be provided from stereochemical considerations. If thyroxine is in fact formed biologically from tyrosine through diiodotyrosine the three compounds must clearly be stereochemically related. The relationships of optically active forms of tyrosine to those of diiodotyrosine were

known; thyroxine however had only been isolated by processes involving alkaline hydrolysis and was therefore available in the racemised condition alone. Progress along this line was therefore held up pending the isolation of thyroxine from natural sources in the optically active state. This object was ultimately achieved by a long and tedious process of enzymic digestion of thyroglobulin (Harington and Salter, 1930); the process could be adapted also to the isolation of optically active diiodotyrosine, and accompanying analytical observations showed that both the thyroxine and diiodotyrosine were, for the most part at least, combined in peptide linkage in the original thyroglobulin. With the natural optically active thyroxine in our hands it was possible to settle the stereochemical point, for on the one hand the thyroxine could be deiodinated to give an optically active thyronine, and on the other an optically active thyronine could be synthesised from natural L-tyrosine by methods which involved no possibility of a Walden inversion. When this was done the two specimens of thyronine were found to be identical (Canzanelli *et al.*, 1934). All this work had been done by 1934, and as the result of it we could say that no facts had been found in conflict with the original theory of the biogenesis of thyroxine, whilst there was cumulative evidence in support of this theory. Nevertheless the evidence remained circumstantial, and direct proof was lacking. The next step forward came some five years later from quite a different direction.

From the date of the original demonstration by Baumann of the occurrence of iodine in the thyroid and its association with physiological activity many attempts had been made to produce artificial substances with similar activity by treating proteins with iodine under the most varied conditions. Although claims, which must now be accepted as having been well founded, had been made, particularly by Abelin, that active products could be obtained, the evidence was not completely satisfying in that the only pure compound that could be isolated from the iodinated proteins was diiodotyrosine, so that the idea that products with thyroid-like activity could be produced by iodination of proteins had been dismissed by most people from their minds. Then in 1939 came the first publication by Ludwig and von Mutzenbecher in which it was claimed that iodination of casein under appropriate conditions led to a product from which thyroxine could be isolated by conventional methods in substantial amounts.

The results claimed by the German workers were so surprising and so much at variance with existing ideas of the chemistry of thyroxine that I confess to having found them unbelievable. I remember my embarrassment when, on meeting the late Franz Knoop a few months before the outbreak of the war, I was asked by him my opinion of the work, especially as it was obvious that he himself, in his capacity as editor, had had some

qualms about the publication of von Mutzenbecher's paper. Perhaps it was partly this meeting which stimulated me to take the matter more seriously; at any rate I did so and we set about the attempt to repeat the work in my own laboratory. To my own great astonishment we had not the slightest difficulty in confirming von Mutzenbecher's results, and thus when shortly afterwards (1939) he published the still more surprising and significant obervation that simple incubation of diiodotyrosine in alkaline solution led to formation of traces of thyroxine we were prepared to accept this with open mind.

The work of von Mutzenbecher has been of the greatest importance in several respects. From the purely practical point of view it has made possible the cheap large-scale production of iodinated proteins with the characteristic physiological activity of thyroglobulin. Such materials have already been used with advantage both in Great Britain and the United States for eliciting the effect of thyroxine in stimulating lactation on cows. From the point of view of my present discussion the more important aspect of the work is the stimulus that it has given to further study of the chemistry of thyroxine formation.

The products obtained by von Mutzenbecher by iodination of casein contained much iodine combined in forms other than thyroxine. Most of this additional iodine was in the form of diiodotyrosine and monoiodotyrosine, that is to say that, apart from the much greater predominance of non-thyroxine iodine in artifically iodinated proteins as compared with thyroglobulin, the general distribution of iodine in the two types of protein is similar. This statement carries the reservation that it applies only to proteins that are iodinated under precisely defined conditions, namely in slightly alkaline solution and with an amount of iodine equal to or only a little in excess of that required for complete substitution of the tyrosine present in the protein. If larger amounts of iodine are employed the products are biologically inactive; no thyroxine can be isolated from them and other compounds, for instance 3:5-diiodo-4-hydroxybenzaldehyde, which are clearly the result of secondary oxidation, make their appearance. It must also be remembered that proteins differ considerably in the amount of thyroxine which can be formed in them under standard conditions of iodination, nor is this amount directly related to the tyrosine content of the protein under experiment. It is obvious that structural factors play a part in the synthesis of thyroxine which occurs in proteins under von Mutzenbecher's conditions of iodination; what exactly these structural factors are can at present only be a matter for speculation.

From our immediate point of view the more interesting part of von Mutzenbecher's work is not that which deals with iodination of proteins but the further observation which I have already mentioned that a small

synthesis of thyroxine occurs when diiodotyrosine is incubated in alkaline solution under aerobic conditions. This finding has been studied extensively by Mrs. Pitt-Rivers, working in my laboratory, and the results that she has obtained are of considerable importance in relation to the general problem of thyroxine biosynthesis, and also to the mode of action of the so-called antithyroid drugs.

Although the coupling of two molecules of diiodotyrosine to give thyroxine can be crudely formulated as a process involving only the elimination of a molecule of alanine, it was at once obvious that the synthesis of thyroxine from diiodotyrosine first observed by von Mutzenbecher in fact involved an oxidation. This was apparent from the facts that the reaction did not occur under anaerobic conditions and that under aerobic conditions it was inhibited by the presence in solution of reducing agents such as thiosulfate and sulfite. It therefore seemed desirable to study the effects on the reaction of variation of the physical conditions, such as temperature and pH and to investigate the action of different oxidising agents added in various proportions.

The action on diiodotyrosine of oxidising agents such as persulfate and ferricyanide, which are known to produce from other para substituted phenols compounds of the type that might be expected to be intermediates in the formation of thyroxine, led to no good result; total destruction of the diiodotyrosine occurred and no thyroxine or other identifiable iodine-containing compound could be isolated from the reaction products. By the use of hydrogen peroxide in large excess at an elevated temperature (about 70°) and by conducting the reaction in a continuously agitated biphasic mixture of water and butanol, it was however possible to obtain considerably larger amounts of thyroxine, the net yield of which was some 5–10 times greater (0.4–0.8%) than could be achieved under the simple conditions employed by von Mutzenbecher. The effect of the pH on the course of the reaction was quite marked, the best yields being obtained at about pH 10, that is to say at a point where about 95% of the carboxylic and phenolic groups of diiodotyrosine would be dissociated (Harington and Pitt-Rivers, 1945).

The experiments that I have just mentioned were of interest in that they provided further direct evidence that the conversion of diiodotyrosine into thyroxine was an oxidative process; in so doing they stimulated thought on the chemical mechanism involved. Even before our own work had been published T. B. Johnson and Tewkesbury (1942) had suggested in a brief note a speculative scheme for the conversion based on a free radical mechanism. The question was further and more elaborately developed by myself (1944) in an argument which took into account the probable relative reactivities of the different ionic species existing in an alkaline solution

of diiodotyrosine. I cannot here however digress from my main theme to go into this matter which is essentially one of theoretical organic chemistry. Rather do I wish to return to the further and more interesting developments of the experimental study of the von Mutzenbecher reaction.

The limit having apparently been reached to the improvement of the yield of thyroxine by direct oxidation of free diiodotyrosine attention was next directed to the behaviour of derivatives of this amino-acid in which the amino-group was protected by acylation, the idea being that with such derivatives side reactions resulting in total destruction of diiodotyrosine or at least in degradation of its side chain might be suppressed. The first experiments of this kind were made with the simple derivative N-acetyldiiodotyrosine and they were immediately successful. When a solution of N-acetyldiiodotyrosine was incubated aerobically at the correct pH a white crystalline precipitate appeared in the flask after about thirty hours; this precipitate increased in amount up to a maximum at about 14 days. When the material was collected and examined it proved to be the pure sodium salt of N-acetylthyroxine; moreover the amount formed represented a net conversion of the acetyldiiodotyrosine into the corresponding thyroxine derivative which was of an entirely different order from that obtainable either under von Mutzenbecher's original conditions or by the hydrogen peroxide oxidation of free diiodotyrosine which I have described. A still further improvement was achieved by the use of a somewhat more complex derivative of diiodotyrosine, namely N-acetyldiiodotyrosylglutamic acid; when this compound was incubated aerobically at the appropriate pH net conversions into the corresponding thyroxine derivative as great as 35% of the theoretical were observed (Pitt-Rivers, 1948).

In the description of these experiments with diiodotyrosine derivatives I have referred more than once to the appropriate pH, and this brings me to a point that I consider to be of considerable importance. It will be remembered that so far as the reaction of the solution is concerned the optimum conditions for conversion of free diiodotyrosine into thyroxine were found at pH 10. With the derivatives of diiodotyrosine however the situation is quite different; in this case the optimum lies at pH 7.5. In these derivatives the diiodotyrosine is combined in peptide linkages similar to those by which it is bound in the molecule of thyroglobulin; it is surely not unreasonable to see some significance in the fact that whilst the free amino-acid requires drastic treatment at high alkalinity for maximal conversion into thyroxine, the amino-acid combined as it is in the thyroid undergoes the same conversion to a far greater extent under essentially physiological conditions, namely at 37° and pH 7.5.

Assuming that the conversion of diiodotyrosine into thyroxine under the conditions described is an oxidative process, it remains to consider what

is the effective oxidising agent. There seems to be little doubt that this is iodine. When diiodotyrosine or its derivatives are kept in slightly alkaline solution they begin almost as once to liberate small amounts of iodine so that the availability of this as an oxidising agent is not in doubt; furthermore mild reducing substances that react with iodine such as thiosulfate and (as we shall see later) many antithyroid drugs inhibit the reaction, which, on the other hand, is accelerated by the initial addition of iodine. Whether the iodine is effective as free iodine or as hypoiodite is difficult to determine under the conditions of the experiment; the distinction is in any case a somewhat academic point.

Taking a general view of the work on the conversion of diiodotyrosine into thyroxine it seems to me reasonable to claim that the experiments of Pitt-Rivers provide an *in vitro* analogy of the process of thyroxine synthesis as we may suppose it to occur in the body. In essence they show that in a system containing tyrosine bound in peptide linkage and free iodine (or hypoiodite) under biological conditions of temperature and pH the synthesis of thyroxine is an inevitable event. In such a system the iodine will have two functions to perform; firstly it must iodinate some of the tyrosine and secondly it must play the part of an oxidising agent in the conversion of the latter into thyroxine; of both these functions we know it to be capable under the conditions prevailing in the body.

If we apply this line of thought to the actual biological process we are faced with the difficulty of explaining how the iodide (which is the form in which iodine is normally absorbed) is converted into the iodine (or hypoiodite) that is required to initiate the series of reactions leading to the synthesis of thyroxine. At present it can only be assumed that the oxidation of the iodide is effected by an oxidising system in the thyroid; several enzymic oxidising systems are known that are capable of bringing about this reaction, although none is known to be specifically associated with the thyroid. Assuming however the presence of a suitable oxidising system, synthesis of thyroxine is to be anticipated in almost any living tissue so long as adequate amounts of iodide are available, since the tissue proteins are practically certain to contain tyrosine in a form of combination in which it can undergo the reactions leading to thyroxine. This view would regard the formation of thyroxine as a general biological reaction and would explain the otherwise very puzzling phenomenon of extrathyroidal thyroxine formation described by Morton *et al.* (1943); it would leave as the specific functions of the thyroid the power of accumulating iodide in high concentration and thus increasing the rate of a reaction that would in any case occur, and the additional power of storing the iodine-containing amino-acids in the form of thyroglobulin. It is of course possible that a second enzyme system in the thyroid participates in the stage of oxidative

coupling of diiodotyrosine to give thyroxine. Some evidence in this direction is given by the experiments of Morton *et al.* (1942) who have shown that in hypophysectomised animals radioactive iodine appears in the diiodotyrosine of the thyroid but not in the thyroxine; this suggests that the second stage of thyroxine synthesis depends on a potentiating system which is inactive in the absence of thyrotrophic hormone. The evidence is weakened however by the fact that the total amount of iodide concentrated by the thyroid in hypophysectomised animals is very small so that the conditions are abnormal. I feel myself therefore that it is better to await more direct proof before dismissing the simpler view, which in my opinion is suggested by the experiments of Pitt-Rivers, that the only enzyme system required is one which will oxidise iodide.

In discussing the various lines of work that have thrown light on the biosynthesis of thyroxine and that have a bearing on its mechanism I have departed from chronological order and have referred to work which has been done since the final proof that the process I have discussed actually occurs in the body. It is now some ten years since this proof was provided by the work of Chaikoff and of Leblond and their co-workers (see Perlman *et al.*, 1943; Leblond and Süe, 1941). The essential requirement for obtaining direct evidence was the possession of a method of tracing the course followed by iodine from its introduction into the body to its incorporation in the thyroid hormone; such a method came to hand with the availability of the radioactive isotope of iodine, ^{131}I, and numerous experiments by the workers already mentioned and by others have left no doubt of the course of events. If radioactive iodide is administered to a normal animal it is rapidly accumulated in the thyroid; in a few hours it is mostly in organic combination in the form of diiodotyrosine and as time goes on the concentration of radioactivity builds up in the thyroxine. Here therefore we have a perfectly clear picture of the occurrence in the body of the series of reactions which was first postulated so long ago and in support of which so much ancillary evidence has been produced. We can take our stand on the theory of the biosynthesis of thyroxine as a firmly established fact, and we can use it with confidence in the interpretation of departures from the normal biochemical working of the thyroid, whether these be caused by deficiency, disease or artificial means. It is to some of these questions that I now wish to give attention.

There are in theory several ways in which the normal formation of thyroxine in the thyroid can be interfered with. Firstly there may be a deficiency of the essential element iodine; secondly there may be adequate iodine but a failure of the mechanism by which the iodine is accumulated in the gland; thirdly both supply of iodine and uptake by the gland may be normal but the actual synthesis of thyroxine may be blocked; of all these

states of affairs it it now possible to give examples. Before going on to discuss these it is necessary however to remind you of the outstanding discovery in thyroid physiology, as distinct from biochemistry, that has been made during the period with which I am dealing. This is the discovery that the function of the thyroid is under the control of the thyrotrophic hormone, one of the secretions of the anterior pituitary gland, and that the output of this hormone is itself controlled by the amount of circulating thyroxine. Thus under normal conditions, we have a self-regulating system in which the thyroid and the anterior pituitary exercise reciprocal control of one another.

It is this reciprocal thyroid-anterior pituitary relationship that is the basis of the effects on the thyroid of any of the means of interference with its normal biochemical function that I have mentioned. In all cases the amount of circulating thyroid hormone is reduced; this causes increased output of thyrotrophic hormone which in turn stimulates the thyroid to overactivity and has the physical effect upon it of producing an enlargement or type of goitre characterised by hyperplasia of the secreting tissue of the gland. The simplest illustration of this is to be found in the effects of restriction of iodine intake and the clearest demonstration of the phenomenon is given by the observations of Marine; working with dogs under conditions of natural or imposed iodine deficiency he was able to show that there was a reciprocal relationship between the iodine content of the thyroid gland and the degree of hyperplasia of the glandular epithelium, the lowest iodine content consistent with normal structure being about 1 mg. per g. of dried tissue. The interpretation offered by Marine was based on the not very satisfying conception of a "work hypertrophy"; as I have just indicated however his results are really to be explained by the secretion of excessive amounts of thyrotrophic hormone by the anterior pituitary in response to the decreased thyroid hormone content of the blood.

The possibility of interference with uptake of iodine by the thyroid has often been considered in the past and has been invoked as an explanation of the occurrence of sporadic cases of goitre in circumstances where there is no reason to suspect a deficiency of iodine. Direct evidence that interference of this kind is possible was first provided by the work of Chesney *et al.* (1928) and later of Marine *et al.* (1929) on the goitrogenic effect of a cabbage diet in rabbits. This goitrogenic effect could be antagonised by simultaneous administration of excess of iodine and was therefore ascribed to interference by some constituent of the cabbage with the access to the thyroid of the small amounts of iodine normally present. A good deal more light on the problem has been thrown by studies of the action of thiocyanate.

It was first observed by Barker (1936) that certain patients under treat-

ment with thiocyanate for hypertension developed goitre accompanied by signs of myxoedema. This goitrogenic effect of thiocyanate has since been confirmed in animals and man, and it has also been made clear that it can be neutralised by administration of iodine. This means that synthesis of thyroxine can proceed normally in presence of thiocyanate and that the primary effect of the latter is on the power of the thyroid to concentrate iodine. It is true that some adverse effect on the process of organic combination of iodine in the thyroid (i.e. thyroxine synthesis) can also be demonstrated with thiocyanate, but this occurs only with very large doses. More significant is the observation that thiocyanate can bring about a rapid discharge from the thyroid of iodide previously accumulated. It seems indeed that thiocyanate prevents the maintenance of the high concentration gradient that normally exists between the iodide of the thyroid and that of the circulating blood, although how it exercises this effect is quite unknown.

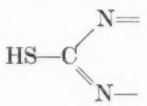
We now come to substances which disorganise the biochemistry of the thyroid by interfering with the actual synthesis of the hormone within the gland, and paradoxically enough the first of these is iodide itself. The fact that some control over the hyperactivity of the thyroid in Graves's disease could be obtained by administering iodide in relatively large amounts was first observed accidentally by Trousseau and was much later developed by Plummer into what became an established method for the treatment of the disease. The theoretical basis for the treatment remained almost non-existent, but modern methods have made it possible to demonstrate that excess of iodide in the blood does prevent the organic binding of iodine in the thyroid; moreover the synthesis of thyroxine by thyroid slices respiring *in vitro* is inhibited by addition of iodide to the medium. These observations leave no doubt as to the objective reality of the depressing effect of iodide on thyroid function, but they do not provide an explanation of the phenomenon. It has been suggested that the effect is due to inactivation of the thyrotrophic hormone, which, as shown by Rawson (see Albert *et al.*, 1946, 1947) is easily brought about by iodine. This inactivating effect however is only brought about by free iodine, and it is not easy to see why the iodine should inactivate the thyrotrophic hormone rather than undergo the normal process of incorporation into diiodotyrosine and subsequently thyroxine. On the other hand according to Li (1942) the rate of iodination of tyrosine at the biological pH of 7.4 is governed by the concentration of hypoiodite in the system; since the reversible reaction of iodine with water is expressed by the equation

$$I_2 + H_2O \rightleftarrows HIO + H^+ + I^-$$

it is clear that the greater the concentration of iodide the smaller will be

that of hypoiodite, so that the iodination of tyrosine would be depressed by high iodide concentrations. To me as a biochemist this simpler explanation of the thyroid-inhibiting action of iodide has the greater appeal.

Much more powerful than iodine in their effects are the antithyroid compounds that have been discovered in recent years. This great development originated in the almost simultaneous observations of Mackenzie and Mackenzie (1943) and of Astwood (1943) that prolonged administration to rats of certain sulphonamides and of thioureas caused hyperplasia of the thyroid with loss of colloid and iodine and simultaneous reduction of metabolic rate. The overall effect was similar to that of thiocyanate, but there was a sharp distinction in that the new antithyroid substances were not antagonised by iodine although their effects could be neutralised by simultaneous administration of thyroxine; there was no evidence that they affected the peripheral action of thyroxine and it was therefore clear that their action must be on the process of synthesis of thyroxine within the gland. There is no need for me here to go over the familiar story of the rapid development of these observations into a method for the treatment of hyperthyroidism which has become an established part of medical practice; before proceeding to speak of the mechanism of action of antithyroid compounds of this type I should however remind you that, with one exception, the more active members of the group, including the commonly employed thiouracil derivatives, contain the grouping



and may therefore be regarded as derivatives of thiourea; the exception is the naturally occurring goitrogenic compound recently isolated by Greer and Astwood (1948) from the yellow turnip.

The great amount of work that has been done with the aid of modern techniques, particularly by the use of radioactive iodine, to trace the metabolism of iodine within the thyroid, has amply confirmed the indications of the early experiments as to the general mode of action of the thiourea type of antithyroid agent. As to the more intimate details of their mode of action there has been much speculation but there is little certainty. An obvious explanation of the effect would be inhibition of the oxidising enzyme system responsible for the liberation of iodine from iodide; it is still possible that this is the true explanation, but there is no conclusive evidence. The nature of the enzyme system catalyzing the oxidation of iodide is in any case in doubt as I have already pointed out. The cytochrome-cytochrome oxidase system, which might well be responsible

for the oxidation, is not inhibited by the thiourea derivatives, whilst peroxidase, which undoubtedly can oxidise iodide and is inhibited by the thioureas has not been demonstrated with certainty in the thyroid; the same remark applies to tyrosinase. The single enzymic experiment which might be thought to represent the course of events in the thyroid is that of Keston (1944) who demonstrated the iodination of casein by iodide in presence of xanthine oxidase, and the inhibition of this reaction by thiourea. This line of thought and experiment has therefore not taken us far.

In this case again considerable weight is in my opinion to be attached to a relatively simple chemical theory which was first advanced by Landgrebe (see Campbell *et al.*, 1944) who suggested that the inhibitory action of thiourea depended upon its reactivity with iodine; a similar reactivity was later demonstrated for thiouracil by Miller *et al.* (1945). This theory has been further developed by Pitt-Rivers (1950) who has provided additional evidence in its favour by showing that not only is the *in vitro* synthesis of thyroxine under the conditions which I have described inhibited by thiourea derivatives, but that the degree of inhibition is related to the antithyroid activity in any given case studied, with one exception, namely ergothioneine. According to this hypothesis therefore antithyroid agents of the thiourea type exercise their effects by keeping the iodine in the reduced condition and thus preventing its combination with tyrosine.

Clearly this theory cannot be regarded as firmly established. There are other factors to be considered concerning which there is no certain evidence, in particular the question of the tendency of various substances to build up high concentrations in the thyroid. Differences in this respect between antithyroid compounds would be reflected in corresponding differences in antithyroid effect and might well account for the anomalous behaviour of ergothioneine. Moreover, although the theory explains the action of the thiourea derivatives, it does not so easily explain the qualitatively similar but much weaker action of compounds such as the sulphonamides and *p*-aminobenzoic acid. Nevertheless it is attractive in its simplicity, and further thought and work along these lines is highly desirable.

Before I conclude there is one other point on which I should like to touch briefly: this is the nature of the circulating thyroid hormone. It is strange that at this stage in thyroid research such an elementary question should still be a subject for discussion, but there have been many facts that have militated against the acceptance of the simple view that the circulating hormone is thyroxine. In the first place, although all the characteristic effects of the thyroid can be produced with thyroxine, there is a delay in its action which at first seemed difficult to explain. Secondly it has never been possible to demonstrate satisfactorily a direct action of thyroxine on isolated tissues in "acute" experiments. Thirdly some analytical

evidence tended to show an association of physiological activity with total iodine rather than with thyroxine-iodine of the thyroid. These considerations led many people to the thought that the real hormone might be thyroglobulin and that thyroxine was essentially an artefact of the method of isolation.

On two grounds it always seemed to me unlikely that thyroglobulin could be the hormone. On the one hand immunological identification of thyroglobulin in the blood of the thyroid vein was only possible under the abnormal conditions following partial thyroidectomy; on the other thyroglobulin was known to be fully effective when given by mouth to a thyroidectomised animal, in which it was scarcely conceivable that re-synthesis could occur after the breakdown which must have preceded absorption. I did however myself incline for many years to the view that the natural hormone might be a peptide derived by partial breakdown of thyroglobulin and perhaps containing both thyroxine and diiodotyrosine.

More recently I have come to feel that this conception is an unnecessary complication; the reasons for the change of view are several. So far as the puzzlement about the delayed and prolonged action of thyroxine is concerned, it must be remembered that this arose in the days when one thought of hormonal action in terms of the rapid and specific effects of such hormones as secretin and adrenaline; since then the knowledge acquired of the action of the sex hormones and the anterior pituitary hormones has led to a complete revision of thought on hormonal action. The same consideration applies to the failure to elicit direct action of thyroxine on tissues in "acute" experiments. Furthermore the analytical evidence apparently relating physiological activity to total iodine content was slender and was based on unsatisfactory methods for determining thyroxine. Finally, and most important, we now have the direct demonstration by the experiments of Taurog and Chaikoff (1948), Gross, Leblond *et al.* (1950), and of Laidlaw (1949) that thyroxine is present as such in the blood. I think therefore, in spite of some conflicting evidence such as that brought forward by Salter & Johnston (1948), that it is most reasonable at present to accept the view that thyroxine is the true hormone and in no sense an artefact.

This then is the picture that I have in my mind of the biochemistry of the thyroid as it has developed during the last quarter of a century. I am fully conscious that it is an incomplete picture; there are aspects which I have left untouched or which I have barely mentioned. At the same time what I have said may give some idea of the progress that has been made and I would like to think that it may illustrate the importance in a complex development of an underlying conception.

Much of what I have said may be taken as established fact, but perhaps even more lies still in the realm of hypothesis. If a successor in this Lecture-

ship should chance to choose the same subject in 25 years' time he will doubtless have a very different picture to present. Of one thing alone we can be certain, and that is that the continuing interest in the problem will be such as to ensure great progress. The future lecturer will have plenty to talk about if he can answer the questions that I have left open; who can say that he will not have something to discuss that we have not yet dreamed of?

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NOTES AND COMMENTS

ESTROGENIC ACTIVITY OF ESTRIOL IN DIFFERENT VEHICLES AND DIFFERENT ANIMALS

Contradictory results have been published on the estrogenic effect of aqueous and oily solutions of estriol in normal and spayed female mice and rats. Curtis and Doisy (1931) found estriol to be half as active as estrone. This result has been confirmed by Marrian *et al.* (1931, 1932, 1934), but it was challenged by Huffman and Grollman (1947). There is, however, a marked difference in activity depending upon the vehicle used for injection, an oily solution of estriol being more active than an aqueous one. This has been shown by Butenandt *et al.* (1931, 1932, 1933), Cartland *et al.* (1935), Meyer *et al.* (1936). Burn and Elphick (1932) were the only investigators who found estriol more active in aqueous than in oily solution.

Perusal of the literature also shows that estriol is believed to be more active in intact animals than in spayed ones. It has been assumed that this finding supports the view that the ovary transforms estriol into estrone. Browne (1933) claimed that an intact infantile rat requires less estriol than a spayed one for obtaining the estrogenic effect The same has been claimed for Emmenin, the estriol glycuronide recovered from the placenta by Collip *et al.* (1934). Huffman and Grollman, however, found no difference between the estrogenic activity of estriol injected into intact immature rats and into castrated adult rats.

In view of these discrepancies we have studied the estrogenic activity of estriol in oily and aqueous solution in intact and spayed rats and mice.

TECHNIQUE

We determined the estrogenic effect of estriol* in 200 animals: 50 spayed and 50 intact female mice, and 50 spayed and 50 intact female rats. Injections were administered in 3 doses over a period of 36 hours. Smears were taken every 12 hours from the 48th through the 96th hour after the first injection. For the assay, estriol was dissolved either in olive oil or in n/100 NaOH with 10 per cent alcohol.

RESULTS

The results are presented in Table 1. The assumption that estriol is more active in the intact female animal than in the spayed one was not borne out. Furthermore, estriol is shown to be 10 times more active in oily than in aqueous solution.

Adult castrated female mice (weighing 20–25 g.) required the same quantity of estriol (10 gamma in aqueous solution and 1 gamma in oily solution) as infantile intact mice for the induction of the estrus reaction. Adult castrated female rats (weighing 150–160 g.) require 200 gamma of estriol in aqueous solution for full estrus reaction, whereas infantile castrated female rats (weighing 30–35 g.) require only 100 gamma; the difference in requirement being due to the considerable difference in weight of the animals. This was proved by the estrus reaction in infantile intact female rats (weighing 30–35 g.), which reacted to 100 gamma as well. Similar weight proportions prevailed in rats when estriol was given in oily solution.

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* We are indebted to Dr. Oliver Kamm (Parke, Davis & Co., Detroit) for supplying us with estriol.

TABLE 1. ESTROGENIC EFFECT OF ESTRIOL IN SPAYED AND INTACT FEMALE MICE AND RATS

Animal	Weight	Vehicle	Estrogenic activity: 1U
A. *Experiments with mice*			
Adult castrated female mice	20–25 g.	auqeous	10 gamma
Infantile intact female mice	12–15 g.	aqueous	10 gamma
Adult castrated female mice	20–25 g.	oily	1 gamma
Infantile intact female mice	12–15 g.	oily	1 gamma
B. *Experiments with rats*			
Adult castrated female rats	150–160 g.	aqueous	200 gamma
Infantile castrated female rats	30–35 g.	aqueous	100 gamma
Infantile intact female rats	30–35 g.	aqueous	100 gamma
Adult castrated female rats	150–160 g.	oily	15 gamma
Infantile castrated female rats	30–35 g.	oily	10 gamma
Infantile intact female rats	30–35 g.	oilu	10 gamma

SUMMARY

1. There is no difference in the reaction of spayed or intact female infantile rats or mice towards estriol, the estrogenic effect being obtained in mice with 1 gamma and in rats with 10 to 15 gamma in oily solution. Hence the ovaries do not seem to play any rôle in the alleged conversion of estriol into estrone within the body.

2. Aqueous solutions (n/100 NaOH+10% alcohol) require ten times the amount of oily solutions (viz. 10 gamma in mice and 100 to 200 gamma in rats) for the production of the estrogenic effect. Adult rats require a higher dose (200 gamma) than infantile rats (100 gamma) in accordance with their higher weights.

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THE MECHANISM OF SYMPHYSEAL RELAXATION CHANGES IN PROTEINS OF THE GUINEA SYMPHYSIS DURING PREGNANCY[1]

In a previous paper (Frieden and Hisaw, 1951), attention was called to alterations in connective tissue proteins of the symphysis pubis of guinea pigs treated with estrogen and relaxin. It was shown that relaxation was accompanied by an increased sensitivity of the water-insoluble proteins of symphyseal tissue to alkali and to trypsin, with the effect of the latter reagent being most striking. Collagen N (NaOH or trypsin-resistant N) ranges from 65–78% of the total water-insoluble nitrogen of the estrogen treated, but unrelaxed symphysis, whereas in relaxed animals the corresponding range is from 50–68%. These studies have now been extended to include the relaxed symphysis of the pregnant guinea pig.

EXPERIMENTAL

The data presented here were collected on a total of thirteen animals. Of these, one aborted at an early stage of gestation, and was later used as a control, as was another animal which was found to be non-pregnant at autopsy. Three animals were post-parturient. Approximate duration of pregnancy in the others was determined from the date of the last estrus.

Symphyseal tissues from control animals (weighing 40–60 mg.) and those from animals pregnant less than 54 days (weighing 65–300 mg.) were divided into two groups, approximately similar with respect to duration of pregnancy, for treatment with alkali or trypsin, respectively. Those weighing more than 500 mg., from animals nearly at term and including one post-parturient specimen, were divided longitudinally; one half was extracted with alkali and the other treated with trypsin. The subsequent procedure was essentially as described previously (Frieden and Hisaw, 1951). Extraction with alkali was for an initial period of 39 hours at room temperature, followed by a second extraction for one hour. Trypsin-treated tissues were incubated for 39 hours at 37° C. In accordance with previous convention, the proportion of total nitrogen extracted by dilute (0.9%) salt solution is designated as "soluble N." Calculations of the proportion of total insoluble N removed by trypsin and alkali are included in the summary of the data (Table 1).

RESULTS

The proportion of nitrogen found to be soluble in saline is considerably lower, for animals in the later stages of pregnancy, than the corresponding figures for animals in which the symphysis was relaxed by the administration of relaxin. This difference appears to be due to the fact that the appearance of extravasated blood in the symphysis, which is of frequent occurrence in relaxin-injected animals, is less pronounced in the pregnant animal.

The data on collagen content (NaOH- and trypsin-resistant N) of the symphyses of non-pregnant animals or those in the early stages of pregnancy (C_2, C_4, R_{74}, R_{69}) are in good agreement with the values observed previously in estrogen-treated, but unrelaxed animals. Table 1 also indicates that the relaxation of the guinea pig symphysis during pregnancy is characterized by changes in collagen which correspond to those observed in experimentally relaxed animals. Our previous studies indicated that changes in trypsin-sensitivity of the connective tissue proteins are manifested at an earlier stage in the phenomenon of relaxation than are the changes in alkali sensitivity; in the present ex-

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TABLE 1. EFFECTS OF TRYPSIN AND ALKALI UPON THE SYMPHYSEAL TISSUE OF PREGNANT GUINEA PIGS

Animal No.	C_4	C_3	R_{74}	R_{69}	R_{71}	R_{78}	R_{73}	R_{68}	R_{67}	R_{72}		R_{57}		R_{66}		R_{76}
Days after mating	0	0*	27	43	48	50	54	54	—†	59		59		P‡		P§
Body weight, gm.	495	520	493	735	830	873	710	720	—	915		970		515		—
Pad weight, mg.	42	61.5	65	165	233	312	224	318	1245	578		536		746		545
Total N, mg.	1.10	2.07	1.97	3.63	4.87	5.96	5.02	6.53	17.52	9.11		11.41		12.86		11.65
% of wet weight	2.62	3.36	3.03	2.20	2.09	1.91	2.24	2.05	1.41	1.58		2.13		1.72		2.14
Soluble N, mg.	0.17	0.39	0.32	0.99	1.38	1.73	1.36	1.60	7.65	3.03		3.51		4.01		5.74
% of total	15.5	18.9	16.2	27.0	28.3	29.0	27.1	24.5	43.6	33.2		30.7		31.2		53.8
Residue N, mg.	0.93	1.68	1.65	2.64	3.49	4.23	3.66	4.93	9.87	2.93	3.15	3.99	3.91	4.67	4.18	5.91
NaOH-soluble N, mg.	0.27		0.49		1.21	1.29				1.27		1.51		1.83		
% of residue N	29.4		29.7		34.6	29.8				43.3		37.8		38.4		
Trypsin-soluble N, mg.		0.53		0.89			1.56	2.15	3.21		1.32		2.00		1.82	3.90
% of residue N		31.5		33.7			42.6	43.6	32.5		42.0		51.1		43.5	66.0
Collagen N																
% of residue N	70.6	68.5	70.3	66.3	65.4	70.2	57.4	56.4	67.5	56.7	58.0	62.2	48.8	61.6	56.5	34.0

* Aborted 20 days before autopsy.
† Animal killed a few days before parturition. Pregnancy not dated.
‡ Killed one day after parturition.
§ Killed 3–6 days after parturition.

periments, the alkali-soluble N is again observed to be lower than trypsin-soluble N (See also Fig. 1).

The data for animals R_{48} and R_{66} indicate that the effects of relaxation during pregnancy persists for an appreciable period following the disappearance of relaxin from the blood (Zarrow, 1947). This is in contrast to the effects observed when relaxin is injected, where it is found that after about 18–24 hours, although injections are continued, the symphysis begins to revert to its original condition.

We have also examined the effects of chymotrypsin and pepsin upon the symphyseal

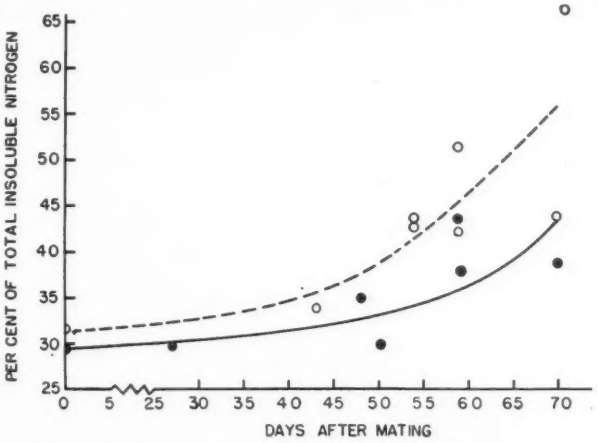

FIG. 1. Effects of trypsin (open circles, broken line) and alkali (closed circles, solid line) upon the water-insoluble proteins of symphyseal tissue of pregnant guinea pigs. The ordinate indicates the fraction of the total insoluble nitrogen dissolved by the reagent.

tissue of guinea pigs treated with estrogen and estrogen and relaxin. Chymotrypsin-resistant proteins of the connective tissue of estrogen-treated animals represent a somewhat smaller fraction ($54.1 \pm 2.7\%$) of the total residue protein than does the trypsin-insoluble material; relaxation does not change this figure. Although essentially all of the connective tissue of both relaxed and control animals is digestible by pepsin (95% in 14 hours), the rate at which digestion proceeds is considerably greater for the former. Thus, while 45% of the total insoluble N of the estrogen-treated, but unrelaxed symphysis, is released in 1.5 hr. at 37°, the corresponding figure for the relaxed animal is 62%.

DISCUSSION

The data presented above, like previous studies on the tissues of the experimentally relaxed animal, are consistent with the assumption that the effects of relaxation are not confined to effects upon the ground substance of the connective tissue, but involve alterations in the collagenous components as well. However, the exact nature of these changes are still nuclear. The effects of trypsin might be explained as secondary to a disaggregation of the collagen fiber to smaller fibrils; it seems unlikely, however, that the effects of exposure to dilute alkali, especially upon the symphyseal tissue of the pregnant animal, can be explained on this basis. The effects noted may more reasonably be explained by assuming that the effect of relaxin is exerted upon the collagen fiber

itself, in such a way as to increase its reactivity to the reagents employed. The hypothesis might be evaluated by careful chemical analysis, for the components of collagen, in residues and digests of relaxed and unrelaxed tissues. It can also be anticipated that electron-microscope studies of such tissues will be of great assistance in the eludication of the mechanisms involved.

SUMMARY

Chemical studies of the connective tissue proteins of the symphysis pubis of the pregnant guinea pig show changes analogous to those observed in the experimentally relaxed animal, namely, an increased sensitivity of the collagenous component to the action of trypsin and alkali.

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THE EFFECT OF THYMUS AND MUSCLE FEEDING UPON GROWTH AND THE CONCENTRATION OF RADIOIODINE I^{131} IN THE THYROID AND OTHER TISSUES OF THE RAT*

Kjerulf-Jensen in 1947 reported that cellular hyperplasia of the thyroid could be produced in rats by adding calf thymus to the standard dietary regime. The present study was conducted in an attempt to confirm this report by study of I^{131} utilization under rigidly controlled conditions.

PROCEDURE

Three groups of 20 Sprague Dawley male albino rats, 40 to 60 grams in weight were maintained for 20 days on a modified Food Research Laboratory Diet.† The diet was pair fed and food consumption and body weights were determined three times each week.

For Group I rats, 40 grams of desiccated calf muscle‡ were added to 960 grams of this

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†

Whole wheat, ground	60.0%
Klim	30.5%
Dried yeast (inactivated, SB)	5.0%
Salt (0.01% KI)	2.0%
$CaCO_3$ (USP)	1.0%
Dried Liver Extract (USP)	1.5%

‡ The desiccated muscle and thymus were prepared by the Viobin Corporation at Monticello Illinois. The frozen tissues of calves of indeterminate age were ground and immersed in ethylene dichloride and the fat filtered off and rewashed to remove all traces of residual fat. The dehydrated, defatted tissue was carefully dried under vacuum to remove all traces of the solvent.

TABLE 1. MEAN WEIGHT GAINS AND DIET CONSUMPTION

Intake per rat	Days	Group I Stock diet plus dried muscle	Group II Stock diet plus dried thymus	Group III Stock diet only
Total food	1–20	204 gms.	198 gms.	209 gms.
Total food	21–90	1043 gms.*	1134 gms.	1186 gms.
Thymus or muscle	21–90	41.7 gms.	45.3 gms.	
NaCl from stock diet	21–90	20.9 gms.*	22.7 gms.	23.7 gms.
NaCl per day	21–90	298 μg.	325 mg.	339 mg.
KI per day (from stock diet)	21–90	29.8 μg.	32.5 μg.	33.9 μg.
Weight gain per rat	1–20	89 gm.	96 gm.	93 gm.
Weight gain per rat	21–90	198 gm.*	207 gm.	225 gm.
Grams of weight gain per gm. diet Consumed:	1–20	0.46 gm.	0.49 gm.	0.44 gm.
	21–90	0.19 gm.	0.18 gm.	0.19 gm.

* Significantly different from Group III value, with a probability of less than 0.01.

stock diet after the rats had received the diet alone for 20 days. For Group II rats, 40 grams of desiccated calf thymus was added to 960 grams of the stock diet after 20 days. Group III rats were continued throughout the course of the experiment on the stock diet alone.

Three hours prior to sacrifice the animals were injected intraperitoneally with 200 microcuries of carrier-free radioactive iodine I^{131}§ in 1 to 2 ml. of physiologic saline solution. The animals were decapitated and the entire pituitary, thyroid, thymus, adrenals, and a portion of the hamstring muscles were taken. A weighed portion of the weighed whole organ was prepared for radioactive assay by a method previously described (Boatman and Moses, 1951) and the remainder taken for histological examination.

The concentration ratios of I^{131} were determined by dividing the per cent radioactivity in each organ by that organ's percentage of body weight (Boatman and Moses, 1951; Boatman, Sunder and Moses, 1951). These ratios are independent of body weight and the weights of the organ and are considered to be a measure of the tissue's ability to concentrate I^{131}.

RESULTS

Table 1 indicates that the rats fed desiccated muscle (Group I) consumed significantly less diet and had a weight gain below that of the control animals (Group III). The animals fed desiccated thymus (Group II) showed a decreased weight gain com-

TABLE 2. MEAN RADIOACTIVE IODINE I^{131} CONCENTRATIONS IN THE TISSUES OF RATS FED DESICCATED THYMUS AND MUSCLE AND IN THOSE FED STOCK DIET ALONE

Tissue	Group I Stock diet plus dried muscle			Group II Stock diet plus dried thymus			Group III Stock diet only		
	% I^{131} Activity	% Body Wt.	I^{131} Conc. Ratio	% I^{131} Activity	% Body Wt.	I^{131} Conc. Ratio	% I^{131} Activity	% Body Wt.	I^{131} Conc. Ratio
Thyroid	.390	.007	52.635	.419	.007	57.411	.501	.008	65.000
Pituitary	.004	.003	1.519	.007	.003	2.310	.004	.003	1.379
Adrenals	.007	.013	.556	.011	.013	.822	.009	.012	.732
Thymus	.055	.148	.374	.053	.140	.375	.055	.140	.390
Muscle	.146*	.335*	.434	.135*	.309*	.438	.220*	.267*	.823

* On the basis of 1 gram of tissue.

§ I^{131} supplied by the Oak Ridge National Laboratory, Oak Ridge, Tenn.

pared to the control Group III, but the mean differences between Groups II and III were not statistically significant.

In Table 2 the probability values of the mean differences of per cent body weight, per cent radioactivity and concentration ratios were not statistically significant, offering only a consistent suggestive trend.

There were no discernible variations from the normal in the microscopic appearance of the organs studied in any of the three groups.

DISCUSSION

No evidence from the data of this study was found to support the conclusion that the administration of desiccated thymus or muscle from young calves fed to young growing rats produced histological or functional evidence of altered thyroid physiology.

Differences in gain in body weight seen between Group I rats, fed desiccated calf muscle in stock diet, and Group III, fed stock diet alone, can be explained on the basis of amount of diet consumed. It should be noted that the greater increases in body weight of the control Group III may be due to a significantly higher salt intake and resultant increased water retention.

SUMMARY

No significant differences were observed in rats fed 4% desiccated muscle or thymus in the ability of the thyroid, pituitary, adrenals, thymus or muscle to concentrate I^{131} or in the histological appearance of these tissues compared to control animals. Differences in weight gain were attributed to differences in salt intake in the diets.

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COMPARATIVE ESTROGENIC AND PITUITARY DEPRESSANT ACTION OF PARA-HYDROXYPROPIOPHENONE IN THE RAT

Recent reports have indicated that although para-hydroxypropiophenone (PONE) is a weak estrogen it is disproportionately potent as a pituitary depressant (Buu-Hoi *et al.*, 1950; Perrault, 1950). Because of the potential therapeutic value of a compound with such properties, we have undertaken a bioassay of the respective estrogenic and pituitary depressant action of PONE[1] using estrone as a basis for comparison. For a test of estrogenic activity, we have employed a modification of the method of Lauson *et al.* (Lauson, 1939). The pituitary effect was determined by the use of the female parabiotic

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[1] We are indebted to Dr. Richard Tislow, Schering Corp. for our supply of crystalline para-hydroxypropiophenone which was procured from Laboratoires Laroche-Navarron, Paris, France.

rats according to the technique described by Meyer and Hertz (Meyer & Hertz, 1937). In this procedure, a pubescent ovariectomized rat is placed in parabiosis with an intact female partner. The degree of subsequent hypertrophy of the ovaries of the intact female may be used as an indicator of the gonadotrophic activity of the pituitary of the castrated animal. It has been repeatedly demonstrated that this gonadal hypertrophy can be quantitatively inhibited by the administration of estrogens and androgens (Kallas, 1930; Hertz & Meyer, 1937; Meyer & Hertz, 1937 and Byrnes & Meyer, 1951).

TABLE 1. ESTROGENIC POTENCY OF PARAHYDROXYPROPIOPHENONE

Dose* (mgms.)	Uterine weight (mgms.)	Body weight (grams)
0.0	15	45
	17	50
	18	53
	21	48
1.0	29	63
	23	47
	36	54
	29	60
2.5	43	56
	50	48
	56	62
	53	60
5.0	108	62
	88	51
	99	48
	83	51

* Total dose given in 3 daily subcutaneous injections in total volume of 0.75 cc. corn oil.

MATERIALS AND METHODS

Crystalline para-hydroxypropiophenone was dissolved in corn oil. All injections were given subcutaneously daily in volumes of 0.25 cc. or 0.2 cc. as indicated. Estrone was administered in 0.5 cc. of an aqueous solution daily. Female rats of the Holtzman-Sprague-Dawley strain were employed. A 20% Casein stock diet supplemented with 0.5% whole dried pork liver was fed ad libitum.

For the estrogen assays, weanling female rats were ovariectomized at 22 days of age and were treated once daily for three days. Twenty-four hours after the last injection the animals were sacrificed and the uteri were dissected out, pressed on blotting paper and weighed to the nearest milligram. Comparison of these uterine weights with those obtained following graded doses of estrone served as a basis for calculating the comparative estrogenic potency of estrone and PONE.

Female rats 30 to 32 days of age and weighing between 65 and 75 grams were surgically joined in parabiosis by the procedure of Bunster and Meyer (1933) except for the use of metal skin clips in place of silk thread. Twenty-four hours before the surgical procedure each animal received 30,000 units penicillin in 0.1 cc. peanut oil intramuscularly. The left partner was ovariectomized at the time of the operation. Injections of the castrate partner with either PONE or estrone were begun immediately after the completion of the surgery and continued daily for ten days. Twenty-four hours after the last injection the animals were sacrificed. The body weight of each partner was determined and the ovaries and uteri were weighed to the nearest milligram. Untreated control pairs were handled in the same manner.

RESULTS AND DISCUSSION

The estrogenic potency of PONE is shown in the data presented in Table 1. The uterine weights thus obtained may be compared with those we have previously obtained with estrone which are in essential agreement with the assay curves of Lauson *et al.* (Lauson, 1939). Such comparison permits the conclusion that PONE is approximately

TABLE 2. EFFECT OF PONE ON OVARIAN HYPERTROPHY IN PARABIOTIC RATS

Treatment of castrated partner	Weight of ovaries (mg.)‡	Uterine weights	
		Castrate partner	Intact partner
No treatment	208	71	166
	115	83	176
	158	59	185
	196	35	249
	115	52	133
	140	32	136
	114	87	132
	211	84	238
	213	132	181
	159	31	131
	188	41	171
Mean	165 ± 38	64 ± 30	172 ± 39
PONE (0.5 mg.)*	168	38	166
	204	86	186
	193	54	168
	125	50	144
	186	58	144
	197	29	142
	162	51	144
	161	42	149
	110	35	143
	147	44	88
	135	69	161
	125	55	117
	137	36	97
	127	34	69
	279	33	183
	166	44	216
Mean	164 ± 41	47 ± 14	145 ± 37
Estrone (0.001 mg.)†	35	132	148
	15	140	47
	31	143	213
	30	103	144
	53	143	110
Mean	33 ± 12	132 ± 15	132 ± 54

* Daily those administered in 0.2 cc. corn oil subcutaneously for ten days.
† Daily dose administered in 0.5 cc. H_2O subcutaneously for ten days.
‡ Control ovaries of 20 rats of the same age and strain averaged 22 ± 5 mg.

0.1% as potent as estrone in estrogenic effect in the rat. This finding corresponds with that of Perrault who reports PONE to be about one-thousandth as potent as hexestrol as judged by the Allen-Doisy test in the rat (Perrault, 1950).

The data in Table 2 indicate that PONE is not to be regarded as an effective depressant of pituitary gonadotrophic activity. Whereas one microgram of estrone nearly completely inhibited the ovarian hypertrophy occurring in the intact parabiont, five-hundred times as much PONE failed to alter the ovarian response to the gonadotrophic stimulation from the castrate partner's pituitary. This would indicate that PONE is even a weaker pituitary depressant than its estrogenic potency would suggest. This conclusion is also supported by a review of the uterine weights of the castrate partners

which were injected with estrone as compared with those found in the parabionts treated with PONE.

The development of a compound of low estrogenic activity and of disproportionately great effectiveness as a pituitary-depressant would be of considerable practical and theoretical significance. Mortimer *et al.* (1951) have recently reported studies on estrolactone and a number of other compounds determining the relative gonadal and pituitary-depressant effect of these substances. Their data indicate that in the case of all substances tested the two biological effects were quantitatively related. From our observations, PONE is apparently no exception to this general rule.

SUMMARY AND CONCLUSIONS

Para-hydroxypropiophenone was found by a rat uterine-weight assay to be about 0.1% as potent as estrone for estrogenic activity. The ovarian hypertrophy, which occurs when an intact female rat is placed in parabiosis with a castrate female, was employed as an indicator of pituitary gonadotrophic activity. By this test, para-hydroxypropiophenone was found to be less than 0.5% as active as estrone as a pituitary depressant. Thus, this compound is not to be regarded as having potency as a pituitary depressant which is out of proportion to its weak estrogenic properties.

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SPECIFIC STAINING OF THE THYROTROPHIC CELLS OF THE RAT PITUITARY BY THE GOMORI STAIN

Gomori (1950) has described an elastic tissue stain which also stains specifically certain of the basophil cells of the rat pituitary. Halmi (1950) investigated the changes in the Gomori stained basophils of the rat pituitary under conditions of specific hormone deficiencies. He found that the Gomori stained cells were not affected by castration or adrenalectomy but were reduced to zero within a few days after thyroidectomy. On the

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other hand, the basophils not stained by the Gomori stain were greatly increased in numbers after both castration and thyroidectomy.

Purves and Griesbach (1951) investigating the pituitary basophils by means of the McManus (1946) periodic acid Schiff reaction for glycoprotein, found that the pituitary basophils of the rat could be differentiated into two functional groups. In one group the intensity of the glycoprotein reaction followed closely the gonadotrophic hormone content of the gland while in the other group of cells the intensity of the glycoprotein reaction was correlated with the thyrotrophic hormone content. From the nature of the specific responses of the two types it was concluded that one type was exclusively gonadotrophic in function, the other exclusively thyrotrophic.

We have applied the Gomori stain to the pituitaries of experimentally treated rats and have correlated the Gomori staining with the McManus glycoprotein reaction. We find that the Gomori stained basophils are those thyrotrophic basophils which have high content of thyrotrophic hormone as judged by the intensity of the glycoprotein reaction. By photographing a section stained by the Gomori stain and then treating it by the McManus technique and rephotographing the same field, we have found that the glycoprotein granules in these cells are specifically stained by the Gomori stain. From this evidence we conclude that the Gomori stain is a specific stain for the thyrotrophic hormone and differentiates it from the gonadotrophic hormone.

The increased numbers of basophils appearing after thyroidectomy which do not stain specifically with the Gomori stain are found by the McManus glycoprotein reaction to be low in glycoprotein content giving merely pale diffuse coloration without visible granules. They differ from the increased numbers of basophils appearing after castration since these give a strong glycoprotein reaction corresponding to the increased gonadotrophic potency of castration pituitaries. The explanation of Halmi's observations is that his beta cells represent thyrotrophic cells with high hormone content. After thyroidectomy, the hormone content in these cells is reduced to a low level within a few days and the Gomori stained cells therefore disappear. The cells classed by Halmi as delta cells include all basophils without a high thyrotrophic hormone content and, therefore, include all gonadotrophic basophils plus those thyrotrophic cells with low hormone content. The increases in the delta cells observed by Halmi occurring after thyroidectomy and after castration are due to different causes. After castration, the increase in the delta cells is due to an increased number of gonadotrophic cells with high glycoprotein content. After thyroidectomy, the increase is due to degranulated thyrotrophic cells with low glycoprotein content.

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